

FUNCTIONAL EVALUATION OF THREE DIMENSIONAL BIOPRINTED LIVER CONSTRUCTS DEVELOPED USING PHOTOPROTECTIVE BIOINK

ANUPAMA SEKAR J

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**SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND
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DIMENSIONAL BIOPRINTED LIVER
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PHOTOPROTECTIVE BIOINK**

A THESIS SUBMITTED BY

Anupama Sekar J

TO

THE SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCE S AND TECHNOLOGY, TRIVANDRUM

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2024

DECLARATION BY THE STUDENT

CERTIFICATE

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The thesis entitled "*Functional Evaluation of Three Dimensional Bioprinted Liver Constructs Developed Using Photoprotective Bioink*" was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

Clearance was obtained for this study from the Institutional Animal Ethics Committee for liver cell isolation and animal experiments.

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Submitted by

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ABBREVIATIONS

3D	: Three-dimensional
AA	: Ascorbic acid
AO	: Antioxidant
CAD	: Computer-aided designing
DCFDA	: Dichloro – fluorescein diacetate
ECM	: Extracellular matrix
FBS	: Fetal bovine serum
FRS	: Free radical scavengers
GelMA	: Gelatin Methacrylamide
IMDM	: Iscove's modified Dulbecco's medium
IRG	: Irgacure 2959
LAP	: Lithium phenyl-2,4,6-trimethylbenzoyl phosphinate
MEM	: Minimum essential medium
NMR	: Nuclear magnetic resonance
NPC	: Non-parenchymal cells
PBS	: Phosphate buffered saline
PI	: Photoinitiator
PVC	: Polyvinyl chloride
ROS	: Reactive Oxygen Species
SEM	: Scanning electron microscope
TE	: Tissue engineering
UHMWPE	: Ultra-high molecular weight polyethylene
UV	: Ultra violet

SYNOPSIS

Liver is the largest visceral organ in the human body that performs many vital functions. Two million deaths every year, which is 4% of all global deaths is accounted for liver disease. As liver disease progress to end-stage, the only effective treatment is liver transplantation. However, due to limited availability of donor livers, studies are focused on tissue engineering approaches to develop donor liver substitute. Tissue engineering aims to develop biological substitutes capable of restoring damaged tissue and tissue-engineered functional liver constructs can be a promising substitute for liver transplantation. In conventional top-down method tissue engineering involves seeding cells over a biocompatible scaffold for them to develop into an equivalent functional tissue. The limitation of this approach is an uneven distribution of cells within the scaffold and the lack of tissue organization. Recently, three-dimensional (3D) bioprinting has emerged as a promising bottom-up assembly approach to fabricate customized tissue constructs by layer-by-layer deposition of cells and biomaterials with precise control over the spatial distribution of cells. Cells, biomaterial and the growth medium form the Bioink, which is one of the important components in 3D bioprinting of living tissues. Hydrogels are extensively been explored as main constituent of bioink, and among them, Gelatin Methacryloyl or Methacrylamide known as GelMA has been widely used for biofabrication of tissue constructs. After Bioprinting the printed construct is stabilized by crosslinking. GelMA has crosslinking property through free radical-initiated photopolymerization under 365 nm ultra violet light in the presence of a photoinitiator. Free radicals produced

during UV exposure can be harmful to the cells. Hence a photoprotective property is required in the GelMA based bioink to safeguard cells during hydrogel formation.

In this context, it is hypothesized that “A new formulation of GelMA based bioink that contains a cocktail of free radical scavenging (FRS) molecules will have the photoprotective property to ensure viability and functionality of bioprinted liver construct”. To test the hypothesis, the following objectives were put forward.

1. Synthesis and characterization of Gelatin based formulations of bioink
2. *In vitro* evaluation of GelMA and printability for 3D bioprinting
3. *In vivo* biocompatibility studies of 3D printed constructs
4. Efficacy evaluation of cell laden construct

The thesis is divided into five chapters - Chapter 1-Introduction, Chapter 2- Literature review, Chapter 3-Materials and Methods, Chapter 4-Results and Discussion, and Chapter 5-Summary and Conclusion.

Chapter 1 introduces the area of research beginning with a description structure and functions of liver, followed by type of liver diseases, their complications, and their impact on society. The current treatment strategies using liver-assist devices, hepatocyte infusion, and liver transplantation are discussed with their advantages and limitations. The relevance of liver tissue engineering is further detailed indicating the scope of 3D Bioprinting and the GelMA based bioink. The research problem is defined in the end of the chapter followed by

research hypothesis and the objectives. The chapter ends with a mention of the scope of the thesis.

Chapter 2 reviews the relevant literature on recent advances in liver tissue engineering. The chapter begins with a brief note on the relevance of liver tissue engineering followed by a thorough literature review on the basic elements of liver tissue engineering, i.e., the cell sources and scaffolds. The various approaches of currently known liver tissue engineering are presented, highlighting their advantages and major limitations. Finally, a detailed review is presented specifically on 3D bioprinting technology and bioinks with an emphasis on evaluation of bioinks for 3D bioprinting.

Chapter 3 contains a structured experimental design elaborating the materials, equipment, and protocols involved in the study. The chapter begins with the synthesis and characterization of Gelatin Methacrylamide (GelMA). The methacrylation of gelatin was analyzed by $^1\text{H-NMR}$ spectroscopy and quantified by ninhydrin and TNBSA assay. Photo-crosslinking of low concentrations of GelMA at 365nm in the presence of Irgacure 2959 as photoinitiator was studied. The properties of GelMA hydrogel like water content, weight loss, and sterilization method were studied. The cytotoxicity and cytocompatibility of GelMA hydrogel were evaluated. A GelMA bioink formulation without cells was analyzed for its rheological characteristics and printability. The stability and degradation of 3D printed multilayer grid GelMA construct of size $10 \times 10 \times 2$ mm was analyzed under physiological conditions. The hemocompatibility of the construct was analyzed using human blood as per ISO 10993-4 guideline. The ability of GelMA bioink formulation for liver bioprinting was evaluated by

bioprinting with HepG2 cells and evaluating its liver specific function. Phototoxicity of GelMA was studied by measuring ROS produced during UV crosslinking. GelMA bioink with antioxidants such as Ascorbic acid and α -Tocopherol (GelMA-G-AO) were formulated and evaluated for its photoprotective property. The antioxidants loaded GelMA bioink formulation was evaluated for its cytotoxicity, cytocompatibility, rheology, printability, stability, morphology and mechanical strength. Liver constructs with rat hepatocytes at high cell density of 5 million cells per construct was 3D bioprinted. The histological structure and liver specific functionality were analyzed over 7 days in culture. To improve the organization of rat liver cells into spheroids within bioprinted construct, the bioink formulation was modified with LAP as photoinitiator for improved crosslinking. The modified bioink was evaluated for its cytotoxicity, ROS assay, rheology, mechanical property and spheroid formation. The 3D printed construct with photoprotective bioink were evaluated for biocompatibility subcutaneous implantation in rat model as per ISO 10993-6 guideline. Finally, the 3D bioprinted liver construct was transplanted into rat liver to study the tissue integration and vascularization.

Chapter 4, presents the results obtained and discusses the results in comparison to reports of similar studies. The ^1H -NMR spectrum of GelMA showed proton peaks corresponding to the methacrylamide group and the mean degree of methacrylation was above 80%. 5% GelMA hydrogel was selected for further studies based on the photocrosslinkability and stability. GelMA showed optimal physical properties and was non-cytotoxic. Based on the temperature sweep analysis, the 5% GelMA formulation was modified with addition of 3%

gelatin denoted as GelMA-G exhibited good rheological properties and excellent printability. The printed multilayer constructs were stable and was hemocompatible. Bioprinted liver constructs with HepG2 cells were viable and exhibited liver specific functions. Phototoxicity analysis in GelMA encapsulated cells showed that there was a significant increase in ROS production as a result of photo-crosslinking whereas GelMA loaded with antioxidant cocktails (GelMA-G-AO) showed a significant reduction in ROS production thereby exhibited photoprotection to cells. The addition of antioxidants did not affect the properties of GelMA bioink. It was non-cytotoxic, cytocompatible and printable. Bioprinted liver construct with primary rat hepatocyte and based on the functional and histological evaluation, that 3D bioprinted constructs with 5M cells/construct (high cell density) outperformed those with 1M cells/construct. The long-term functional performance of the high cell density bioprinted constructs in exhibiting liver-specific characteristics was confirmed by from albumin and urea secretion. Collectively, the results of this study provide evidence for the formulation of a photoprotective bioink for high cell density bioprinting of liver constructs. For an efficient tissue organization within the bioprinted construct, modification with LAP as photoinitiator increased the crosslinking density of GelMA-G-AO and has allowed the hydrogel matrix to self-organize the cells to form spheroids with the bioprinted construct. The modification of GelMA-G-AO bioink with LAP also retained the photoprotective property. Histological analysis of the subcutaneous tissues after implantation of 3D printed GelMA-G and GelMA-G-AO constructs in rats for 28 days showed the presence of foreign body giant cells, a fibrous capsule, and neovascularization in the implanted site, showing a promising

indication for the biodegradation of GelMA hydrogel. Also, the histological examination suggests that both GelMA-G and GelMA-G-AO elicited similar immune response when implanted subcutaneously in rat. Following the bioprinted liver transplant into a rat liver, histological examination revealed that the bioprinted liver had successfully integrated into the host liver and that blood vessels had extended into the bioprinted construct from the host tissue. The tissue repair was evidenced by the presence of microvasculature within the bioprinted liver. It can be suggested that there was no adverse immune reaction to the allogenic cell laden constructs and efficient vascularization was achieved in the transplanted bioprinted liver when examined after 28 days of transplantation.

Chapter 5 summarizes the results and conclusions drawn. A GelMA based bioink with photoprotective property was formulated and evaluated for liver bioprinting. Three-dimensional bioprinting with high cell density using GelMA photoprotective bioink allowed biofabrication of tissue-mimetic functional liver constructs. In conclusion, the study confirms that incorporation of antioxidants in GelMA bioink could protect cells from ROS formed during UV exposure. The addition of antioxidants in the standardized formulation of GelMA bioink did not influence the inherent properties of GelMA and exhibited ideal conditions for liver bioprinting. The bioprinted liver with high cell density forms histomimetic functional liver tissue construct. Subcutaneous implantation of 3D printed material and *in vivo* transplantation of bioprinted liver construct into rat liver suggest that the hydrogel does not cause any adverse effect on host. Thus, the results provide preliminary evidence for developing 3D Bioprinted liver with GelMA-G-AO for clinical translation in future.

Chapter 1

INTRODUCTION

1.1 Liver

A human liver is a roughly triangular-shaped organ that weighs approximately 1.5 kg and comprises ~2% of total body weight, making it the largest internal organ and gland in the body (Jeschke *et al.*, 2018). It is made up of very soft, pinkish-brown tissues covered by a thin capsule of dense connective tissue and this capsule is further covered and reinforced by the peritoneum of the abdominal cavity, which protects and holds the liver in place within the abdomen (Rogers and Dintzis, 2018). Liver functions synchronously with many other organs and contributes to the maintenance of basic homeostatic mechanisms.

1.1.1 Structure of liver

The human liver consists of four lobes (left lobe, right lobe, caudate lobe, and quadrate lobe) containing multiple cell types including hepatocytes (70%), cholangiocytes (1%), endothelial cells (12%), stellate cells (3%) and Kupffer cells (15%) (Vernon *et al.*, 2018) (Miyamura and Nishina, 2018). The functions are mostly undertaken by hepatocytes, the parenchymal cells of the liver. Each liver lobe is made up of thousands of hexagonally shaped lobules, which constitute its functional units. Each lobule consists of a central vein at the vertices of the portal triads. The portal triad consists of a portal vein, hepatic artery and biliary duct and

are seen at the vertices of the hexagon. Within the lobules, the hepatocytes are arranged in cords radiating from central vein to portal triads. In between the cords is a vascular space which is lined by a thin fenestrated endothelium and a discontinuous membrane called a sinusoid. These sinusoids contain Kupffer cells which are the resident macrophage of the liver and stellate cells which are hepatic lipocytes. Between adjacent hepatocytes, a minute space of 1 - 2 μm wide is present known as bile canaliculi. The hepatocyte secretes bile salts into canaliculi which transport the bile to the epithelium-lined bile duct (McCuskey and disease, 2012). Cholangiocytes are the epithelial cells lining the bile ducts. Blood from the intestine flows from the portal vein and arterial blood reaches the hepatic artery mix inside the sinusoid and finally gets into the central vein which is a branch of the hepatic vein (Gordillo *et al.*, 2015, Miyamura and Nishina, 2018).

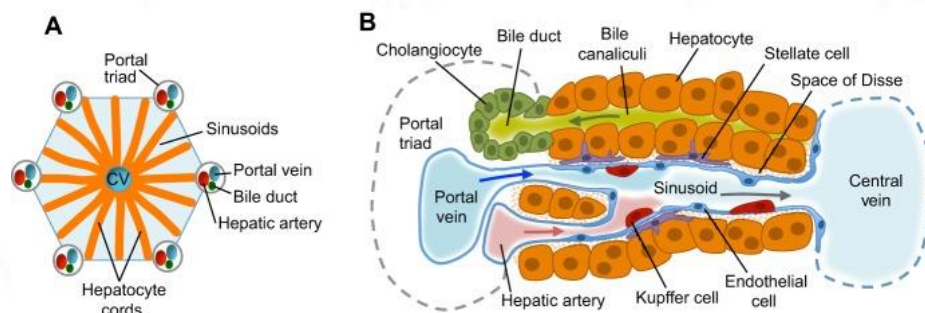


Figure 1. Diagrammatic representation of the cellular architecture of the liver. Reproduced with permission from (Gordillo *et al.*, 2015).

1.1.2 Functions of Liver

The liver is located perfectly between the digestive tract and general circulation, for receiving absorbed nutrients as well as detoxifying absorbed drugs

and other noxious substances (Chiang, 2014). It functions as both an exocrine and an endocrine organ. The exocrine functionality of the liver consists primarily of bile salt synthesis and excretion into the common bile duct, as well as bilirubin conjugation and excretion into the gut. The endocrine functions of the liver include glycemic control via insulin and glucagon. The liver synthesizes important proteins like fibrinogen, albumin, prothrombin, and other amino acids, as well as modifies proteins into enzymes and peptide hormones. The liver also plays a role in fatty acid metabolism and the production of lipoproteins, cholesterol, and phospholipids (Washabau, 2012). It is also involved in carbohydrate metabolism, including glycogen storage and gluconeogenesis as well as in ammonia detoxification into urea (Vernon *et al.*, 2018). In addition, the liver serves as a storage location for vitamin and minerals such as iron. The liver is indeed essential to life, not only due to its complex functions but also due to its role as the blood volume reservoir. Hepatic blood volume appears to range from 25 to 30 mL/100 g liver weight, accounting for 10%–15% of the total blood volume (Lautt, 1977). Thus, the liver serves as an important mediator between the gut and the blood and plays a critical role in the metabolism of macronutrients, hormones, components of blood plasma, and exocrine and endocrine substances (Kalra *et al.*, 2018).

Another important factor that makes liver a unique organ is its innate ability to regenerate which helps in the restoration of function following liver injury. The key to regenerative capability of liver is unlimited proliferative capacity of hepatocytes. Under normal physiological condition hepatocytes are quiescent but when the liver is injured and new liver mass is required to maintain

homeostasis, hepatocytes can be stimulated to proliferate and restore liver function (Wang *et al.*, 2017).

1.1.3 Diseases of the Liver

Liver disease is any condition negatively affecting the normal and healthy performance of the liver. It can be caused due to viral infections, autoimmune disorders, inherited diseases, alcohol consumption, etc. Depending on the duration taken for a normal liver to dysfunction, diseases of the liver can be classified as acute or chronic.

Acute liver failure (ALF), also known as fulminant hepatic failure, is a life-threatening condition where liver function rapidly declines within a few days or weeks due to the death of a significant number of hepatocytes. During the process of converting drugs and toxins into excretable form using the cytochrome P450 (CYP 450) machinery toxic intermediates and by-products may form that can harm the liver (Deborah G. Nguyen^{1*}, 2016). Potential causes of ALF include drug overdose, toxin exposure, and autoimmune hepatitis. ALF can lead to complications like cerebral edema, gastrointestinal bleeding, and kidney failure. The end stage of ALF often leads to symptoms such as jaundice, cerebral edema, bleeding disorders, kidney failure, and infections (Adeva *et al.*, 2012).

Chronic liver disease (CLD) involves the gradual destruction of liver tissue that occurs over months, years, or even decades. This leads to progressive deterioration of liver functions, including the synthesis of clotting factors, and other proteins, detoxification of harmful products of metabolism, and excretion of bile. The spectrum of etiologies is broad for chronic liver disease, which includes

toxins, alcohol abuse for a prolonged time, infection, autoimmune diseases, and genetic and metabolic disorders. The main causes of CLD are hepatitis C virus (HCV), hepatitis B virus (HBV), alcohol-related liver diseases (ARLD), and non-alcoholic fatty liver diseases (NAFLD). Cirrhosis is the final stage of CLD resulting in disruption of liver architecture, widespread nodule formation, vascular reorganization, neo-angiogenesis, and extracellular matrix deposition (Sharma and Nagalli, 2023). The complications of chronic liver disease include variceal bleeding, ascites and spontaneous bacterial peritonitis (SBP), hepatic encephalopathy, hepatorenal syndrome, hepatopulmonary syndrome, and Hepatocellular carcinoma (HCC). When the liver becomes damaged due to complications from acute or chronic liver disease so that it cannot perform its regular functions and undergo normal repair mechanism it is known as End Stage Liver Disease (ESLD) (Tajiri and Shimizu, 2013).

1.1.4 Liver diseases-Global Status

Liver disease is responsible for more than two million deaths globally per year, including those caused by cirrhosis, viral hepatitis, and liver cancer (Higashi *et al.*, 2022). It accounts for 4% of all deaths worldwide, which means that one out of every 25 deaths are due to liver disease, and among them, approximately two-thirds of all liver-related deaths occur in men. Liver cancer alone is responsible for 600,000 to 900,000 deaths. Chronic liver disease is one of the frequent causes of death, especially in the developing world. The increasing prevalence of chronic liver disease has been noted in recent times (Cheemerla and Balakrishnan, 2021). Liver cancer is the sixteenth leading cause of death globally, while cirrhosis is the

eleventh. The most common causes of cirrhosis worldwide are related to viral hepatitis, alcohol, and non-alcoholic fatty liver disease. Around two billion people worldwide consume alcohol, and up to 75 million have alcohol use disorders and are at risk of alcohol-related liver disease. Nearly two billion adults are obese or overweight, and more than 400 million have diabetes, both of which are risk factors for hepatocellular carcinoma and non-alcoholic fatty liver disease. The incidence of viral hepatitis is still widespread around the world, and drug-induced liver damage is becoming a more significant cause of acute hepatitis. Cirrhosis has become the 10th highest cause of death in Africa (previously ranked 13th in 2015), the 9th highest cause in Southeast Asia and Europe, and the 5th major cause of mortality in the Eastern Mediterranean (Devarbhavi *et al.*, 2023). Cirrhosis is also a costly disease. In the United States, liver-related expenses amounted to \$32.5 billion in 2016, with two-thirds of these costs associated with inpatient or emergency department care. Over 20 years, healthcare spending has increased by an average of 4% per year, primarily driven by hospital-based services.

1.1.5 Liver Disease Status

Liver diseases are increasingly being recognized as a significant concern for public health in India. Hepatitis B virus (HBV), alcohol-induced hepatitis, drug-induced liver injury, and hepatitis E virus (HEV) are the main causes of acute hepatitis in adults. HBV is also the primary cause of chronic liver disease. However, there is an increasing prevalence of alcohol abuse, which might eventually surpass HBV as the main factor for CLD. Each year, around one million new cases of liver cirrhosis are diagnosed in India. In 2016, the collective number

of deaths caused by chronic liver diseases and cirrhosis in India was 2.1% of the total mortalities. According to the data released by the World Health Organization (WHO) in 2017, liver disease fatalities in India amounted to 259,749, constituting 2.95% of the total number of deaths. These deaths accounted for 18.3% of all cirrhosis-related deaths worldwide. Hence, according to WHO, liver disease ranks as the tenth leading cause of mortality in India. One out of every five Indians is affected by liver disease, which claims more lives than diabetes and road accidents (Devarbhavi *et al.*, 2023). Patients in India also face significant financial hardships when treating chronic liver diseases, as the cost of diagnosis and intensive care treatment can exceed US\$ 5,000 (Prinja *et al.*, 2018)).

1.1.6 Diagnosis of liver diseases

Clinicians utilize liver function tests (LFTs) to assess the condition of an individual's liver. These tests measure various components, including aspartate transaminase (AST), alanine transaminase (ALT), bilirubin, alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT), which provide information about liver cell damage (S. Sivakrishnan, 2019). Elevated levels of AST and ALT indicate hepatocyte damage, while ALP can indicate damage to the biliary tract. However, it is important to note that ALP can also be present in other tissues like bones. Therefore, when combined with other markers, ALP can provide evidence of hepatocellular injury. A reliable method to evaluate liver function is its capability to produce protein. Although the liver synthesizes an important protein called albumin, its levels cannot solely determine liver performance due to other influencing factors like nutrition status and nephrotic syndromes. Moreover,

albumin has a lifespan of 15 to 20 days, hence it may not indicate immediate liver dysfunction. Hence, albumin levels are combined with coagulation studies to gain an understanding of the liver's functional capacity. Ultrasound is a cost-effective and non-invasive method extensively applied in evaluating the liver. Triple-phase imaging techniques like computed tomography (CT) or magnetic resonance imaging (MRI) are employed to classify and diagnose liver lesions (Kalra *et al.*, 2018).

1.1.7 Current treatment strategy

Although the liver can regenerate and regain all of its mass and function, cirrhosis-induced damage can deteriorate the ability to regenerate, necessitating treatment to address the issue. Depending on the type of liver disease and how far it has advanced, different treatment strategies are offered. In case of viral hepatitis or inherited conditions like Wilson disease, medications are provided whereas for non-alcoholic fatty liver disease, lifestyle changes such as restricted calorie intake and increased physical activities are recommended (Zelber-Sagi and Moore, 2024). Acute liver failure is a critical medical condition that necessitates immediate hospital care. Treatment can potentially reverse certain causes of acute liver failure, while in other cases, a liver transplant may be the sole solution for a cure. End Stage Liver Disease is an irreversible condition, and the only potential solution is a liver transplant. Thus, when the liver progresses to the end stage, transplantation of the liver remains the only option (Li *et al.*, 2021). During transplantation, a diseased or damaged liver is replaced with a healthy one. Three main types of liver transplants can be given to a patient, (i) deceased donor

transplant in which a liver from a recently dead donor is transplanted, (ii) living donor transplant in which a healthy person donates a piece of their liver to the recipient and (iii) split type liver transplant where liver from a deceased individual will be split into right and left lobes and transplanted into two individuals. During orthotopic liver transplantation (OLT), the damaged liver is removed and replaced by a donor organ in the same anatomic location as the original liver. However, patients with severe chronic liver disease having portal hypertension and severe clotting disturbances may find it difficult to dissect and remove the host liver. Therefore, in such cases, heterotopic liver transplantation is carried out where the recipient's liver is left in place and a donor's liver is sewed into an ectopic site (Zhang and Wheatley, 2018). While liver transplantation remains the most effective treatment for liver failure the acute lack of available donors is one of the main obstacles to liver transplantation (Rashidi *et al.*, 2018) contributing significantly to wait for list mortality, the 12-15h short window in which donor organs must be cryopreserved before transplantation (Tas *et al.*, 2021), the lack of a structured database that impedes the matching of matched donors with transplant candidates, the risk of organ rejection, and the requirement for lifelong immunosuppressive therapy, and high morbidity (Ruiz and Kirk, 2015).

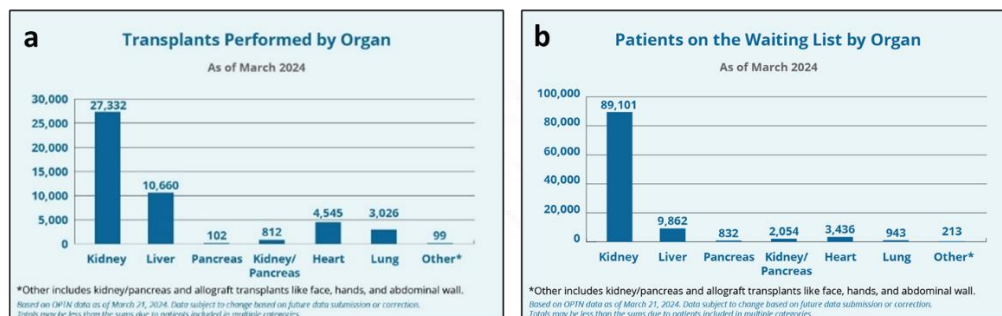


Figure 2. Organ transplantation statistics in India. Data obtained from <http://www.organindia.org>. (a) Graph depicts that liver transplantation is the second most common transplantation performed in India, (b) Graph shows the demand for liver for transplantation.

Extracorporeal liver assist devices (ECLS) are short-term life-supporting devices that regulate blood/plasma parameters to assist the liver function of patients awaiting liver transplantation to provide a temporary solution. In certain situations, with the help of ECLS, complications of liver failure, like hepatic encephalopathy and multi-organ failure, can be delayed, allowing the native liver to gradually regain its functionality. Extracorporeal liver-assist devices can be divided into artificial liver assist (ALA) devices and bioartificial liver assist (BAL) devices. ALA support systems employ a mechanical method without cells to replace the detoxification role of the liver whereas BAL systems use living cells to detoxify blood by separating and running plasma through a hollow-fiber network lined with hepatocytes (Nguyen *et al.*, 2022). ECLS has several drawbacks, such as a lack of accessibility, difficulty in operating, a complex setup of instruments, and movement restrictions brought on by the patient. As a result, alternative therapeutic strategies are required to replace the compromised organ.

The successful results of partial orthotopic liver transplantation indicate that complete liver replacement is not always required to restore liver function

(Rela *et al.*, 2016). This allows the possibility of using hepatocytes as a potential source of cells for liver disease treatment. Cell-based therapy focuses on incorporating functional hepatocytes into the patient's liver through the infusion of hepatocytes into the vascular network, such as the portal vein. Allogeneic hepatocyte transplantation was initially developed as a potential alternative to liver transplantation in which transplanted cells, once engrafted into the recipient's liver, restore the impaired hepatic function. Hepatocytes are primarily obtained from donor livers that are not suitable for organ transplantation due to extended warm or cold ischemia times, mild steatosis, or abnormal anatomy. The procedure for transplantation, which consists of injecting hepatocytes into the liver or spleen, is less invasive compared to whole liver transplantation and can be performed on critically ill patients with a lower level of risk. Over the last two decades, hepatocyte transplantation (HT) has progressed from the bench to the bedside. However, due to poor cell engraftment and time-limited survival of the transplanted hepatocytes, this approach has failed to demonstrate long-term clinical benefits (Yanagi *et al.*, 2017, Mazza *et al.*, 2018).

Therefore, researchers propose to test novel tissue engineering approaches aiming to fabricate three-dimensional functional hepatic tissue in microscale size or whole bioengineered liver to restore lost liver function and address the donor organ shortage issue.

1.2 Liver tissue engineering

Tissue engineering (TE) was defined by Langer and Vacanti in the early 90s as “an interdisciplinary field which applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function” (Vacanti and Langer, 1999). Tissue engineering strategies have the potential to replace, repair, and regenerate tissue/organs for a wide range of biomedical applications including transplantation. It holds out the possibility of generating new tissues and organs through the combination of material scaffolds and a patient’s cells. The initiatives of liver tissue engineering include (1) the generation of whole, implantable, and functional tissue-engineered liver constructs; (2) bioartificial liver systems to prolong the lives of liver patients before liver transplantation; (3) an *in vitro* hepatocyte-based model for drug metabolism/toxicity screening for drug discovery; and (4) for fundamental researchers of liver regeneration, disease, and pathophysiology. Figure 3 illustrates the different methods employed in liver tissue engineering for *invitro* drug screening, disease modeling, and *in vivo* application.

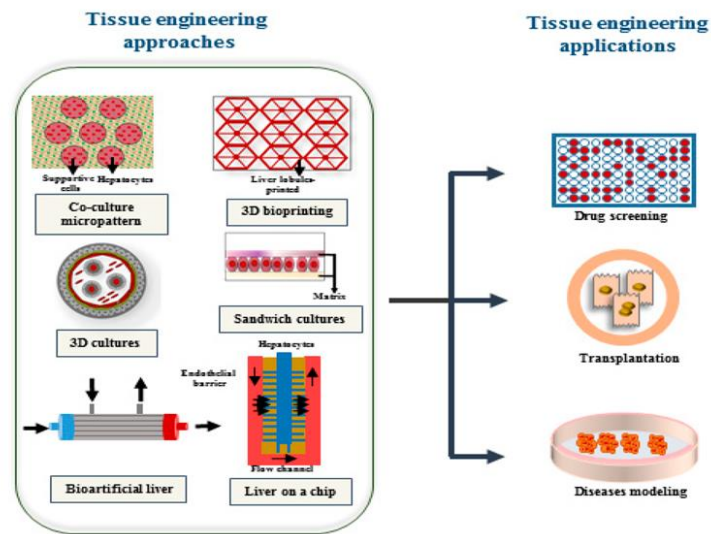


Figure 3. Tissue engineering approaches for *in vitro* and *in vivo* applications. Adapted from (Heydari *et al.*, 2020b)

Liver tissue engineering aims to create transplantable liver tissue *in vitro* with the functionality of the original human liver, allowing patients with liver injury to receive treatment and address the clinical organ shortage (Kryou *et al.*, 2019). The success of tissue-engineered liver construct for *in vivo* application largely depends on the development of liver tissue with the ability to mimic the natural liver architecture and its physiology. In the conventional or top-down tissue engineering method, cells are seeded over a prefabricated porous scaffold and are grown into a functional tissue equivalent. As a result, it poses challenges such as incomplete cell penetration, uneven cell distribution within the scaffold, low cell seeding density, absence of vascularization, and tissue-level cell organization (Wang *et al.*, 2021). Therefore, to address the limitations of the conventional method and improve the transplantation process, a bottom-up or modular approach was introduced. In the bottom-up approach, functional tissues

are fabricated using bioactive molecules, cells or cell aggregates, biomaterials, or hybrid cell-material constructs *via* bioassembly or bioprinting and through subsequent tissue maturation processes (Groll *et al.*, 2018). Bottom-up tissue engineering has unique design flexibility and allows module units to be combined through self- or guided/programmed cell assembly and enables the spatiotemporal modulation of cell-cell and cell-extracellular matrix (ECM) interactions in the engineered tissues.

1.2.1 Three-dimensional Bioprinting

Three-dimensional (3D) Bioprinting is one of the promising technologies, used in regenerative medicine to develop complex tissue structures that mimic native organs and tissues. 3D bioprinting is based on the layer-by-layer precise positioning of biological constituents, biochemicals and living cells, by spatial control of the placement of functional constituents of the fabricated 3D structure (Murphy and Atala, 2014). Based on the CAD model generated from the computed tomographic (CT) image obtained from a patient's damaged or injured organ, 3D bioprinting helps in generating customized biomimetic structures for individual patients (Billiet *et al.*, 2014). The benefit of using 3D bioprinting in the biomedical field is the ability to create complex structures on demand with high precision in a short period of time.

The elements of 3D Bioprinting are bioink, cells, growth factors and bioprinter. The bioink is the primary component of 3D bioprinting which is essential for the generation of functional organs or tissue structures. The bioink, which consists of biomaterials along with cells or cell aggregates in an appropriate

culture medium containing bioactive molecules, is used to biofabricate 3D constructs using a bioprinter. It is important to choose bioinks with ideal properties for the success of bioprinting (Gopinathan and Noh, 2018, Murphy and Atala, 2014). Bioinks that closely resemble a cell ECM and having the optimum gelling properties for bioprinting are the best candidates for enhancing cell function and viability. In this regard, hydrogels are considered as the most prominent biomaterial for 3D bioprinting, mainly due to their similarity with ECM, ability to hold live cells, modifiable chemical structures, tunable mechanical and biodegradation properties (Ramiah *et al.*, 2020). The shape and integrity of the 3D printed constructs are maintained by stabilizing the constructs using either chemical, enzymatic or photo-induced crosslinking (Theus *et al.*, 2020).

Photo-crosslinking is one of the crosslinking method that is most frequently used in 3D bioprinting because of its higher crosslinking efficiency and spatiotemporal controllability in generating stable tissue constructs (Zennifer *et al.*, 2022). In photoinduced crosslinking, hydrogels are formed by polymerization under light irradiation (ultraviolet (UV) or visible light) in presence of photoinitiators. The most commonly used photo-crosslinking method is free radical polymerization. Therefore, the major concern during UV exposure to biological entities is the production of cytotoxic free radicals (Rizzo *et al.*, 2023).

In normal liver, the ECM compartment is less than 3% of the relative area of the liver section and 0.5% of the weight (Bedossa and Paradis, 2003, Lv *et al.*, 2022). Therefore, in liver bioprinting, it is important to fabricate liver construct with high cell density and less ECM in order to mimic the native tissue's structure without compromising on functionality and physiological makeup.

1.2.2 GelMA based bioinks

Numerous hydrogel systems have been explored as bioinks. Among them, modified form of gelatin known as Gelatin Methacrylamide, Gelatin Methacryloyl, or Gelatin Methacrylate all abbreviated as GelMA is a widely used hydrogel for biofabrication of various tissue constructs. GelMA is a functionalized form of gelatin, modified with methacrylic anhydride (Shirahama *et al.*, 2016). The gelatin is a byproduct of the hydrolysis of collagen, which makes up the majority of the ECM in most tissues. Hence, gelatin retains the arginine-glycine-aspartic acid (RGD) sequences that helps in cell attachments as well as the enzymatic degradation sequences of matrix metalloproteinase (MMP) which is crucial for tissue remodeling. The main advantages of gelatin over collagen are better solubility in neutral pH and low level of antigenicity (Yin *et al.*, 2018). In GelMA, the modification with methacrylic anhydride provides gelatin a photo-crosslinking property without losing its biological properties.

The 3D bioprinted constructs with GelMA based bioink can be stabilized by either enzymatic or photo-induced crosslinking. However, it has been noted that, in comparison to GelMA constructs crosslinked with microbial transglutaminase (MTGase), rapid photo-crosslinking aided in maintaining the structural integrity of the GelMA construct (Zhou *et al.*, 2017). In case of GelMA hydrogel formation, methacryloyl-initiated photo-crosslinking is activated by photoinitiators. The selection of photoinitiators is critical for effective polymerization. To produce free radicals effectively, a photoinitiator needs to be highly efficient and have a broad excitation spectrum. 2-hydroxy-1-[4-(2-

hydroxyethoxy) phenyl]-2-methyl-1-propanone (Irgacure 2959) and lithium acylphosphinate salt (LAP) are commonly used photoinitiators which generate free radicals under UV light, and simultaneously participate in chain polymerization to crosslink each other (Ghosh *et al.*, 2023).

1.3 Problem statement

The 3D bioprinted constructs with GelMA bioink are stabilized by exposing under UV and during this time a large amount of free radicals are produced as a result of free-radical initiated polymerization. The generation of free radicals during UV exposure is uncontrollable and has a high potential to interact with the cells encapsulated in the tissue construct. Free radicals can be defined as any molecular species capable of independent existence with an unpaired electron in an atomic orbital. They are highly reactive species that can attack biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids. This can lead to severe cellular damage by interfering with physiological and biochemical processes (Lobo *et al.*, 2010). The cellular damage due to UV exposure can be limited to an extent by selecting a desired wavelength, exposure time, and intensity. However, the real issue arises when the size of the bioprinted GelMA construct increases and it takes longer to crosslink, exposing the cells inside the construct to UV for a longer period of time. Therefore, it is necessary to provide protection to the cells encapsulated in photo-crosslinkable GelMA from cytotoxic free radical attack.

1.4 Conceptualization and formulation of hypothesis

GelMA is a photo-crosslinkable polymer that forms hydrogel through free-radical initiated polymerization. So, under UV exposure, photoinitiator present in GelMA bioink formulation generates free radicals and the radicals are then transferred to the methacryloyl group of GelMA macromers, resulting in covalent bond between two methacryloyl side groups. The number of free radicals generated depends on the concentration of photoinitiator, UV exposure time and GelMA concentration which in turn determines the crosslinking efficiency. Since, the free radicals can induce cytotoxic effect on cells in the bioink it is necessary to control free radical generation without causing significant effect on crosslinking efficiency.

In human body, free radicals are usually produced by regular metabolic processes or due to external factors (radiation, pollutants etc). Some internally generated sources of free radicals are mitochondria, peroxisomes, phagocytosis, inflammation etc. Human bodies reduce the impact of these free radicals by setting up defense mechanism with the help of antioxidants that act as free radical scavengers (FRS). An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, and thereby terminate the chain reaction before vital molecules are damaged. Some of the antioxidants are produced during normal metabolism in the body and some are supplemented through diet (Lobo *et al.*, 2010). Thus, antioxidants can be grouped into two categories: endogenous (enzymatic) and exogenous (non-enzymatic). The enzymatic group includes catalase, the enzymes of glutathione thioredoxin system,

and superoxide dismutase (SOD) and exogenous group includes vitamin C (Ascorbate), vitamin E (α -Tocopherol), carotenoids, flavonoids, polyphenols etc. (Nimse and Pal, 2015).

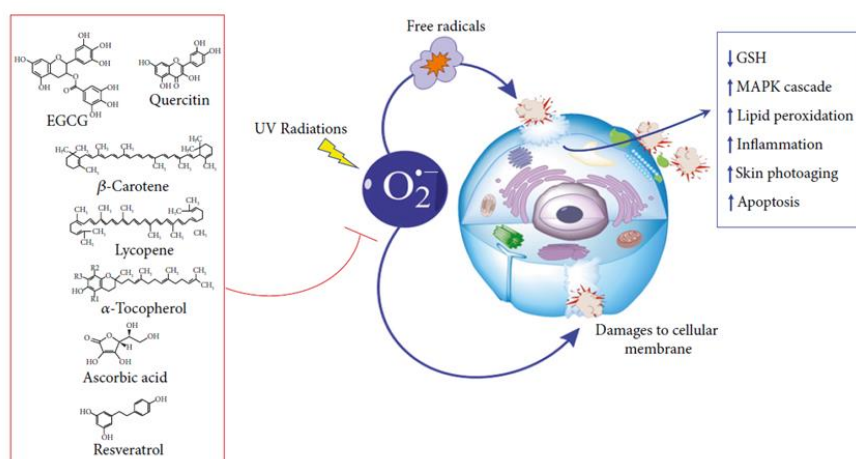


Figure 4. Schematic representation of cellular responses to oxidative stress in the presence (red lines) and absence (blue lines) of antioxidants. Adapted from (Petruk *et al.*, 2018)

In nature, plants as well as human skin are prone to damage by UV rays from the sunlight. UV radiation can induce intracellular Reactive Oxygen Species (ROS) production and leads to deleterious effects on living organisms. Photoprotection is defined as the biochemical processes that assists organisms in dealing with molecular damage caused by solar radiation (Verma *et al.*, 2024). In case of plants, solar radiation affects their photosynthetic machinery and plants use antioxidants like flavonoids and carotenoids for UV protection (Maury *et al.*, 2020). In humans, to protect skin from UV damage, topical applications of sunscreens supplemented with endogenous antioxidants are widely used. The antioxidants that have been studied the most extensively for preventing oxidative damage to the skin are vitamin C, vitamin E, and β -carotene (Jesus *et al.*, 2023).

It has been reported that combining vitamin C and E is better for UV protection to skin than using the same concentration of either vitamin alone. This is due to the fact that vitamin C and vitamin E can interact synergistically and provide enhanced antioxidant protection (Rozman and Gasperlin, 2007).

Vitamin E is a major chain-breaking radical scavenger in the liposoluble compartment, making it the major specific defense line against lipid peroxidation. When a peroxy radical forms in a membrane the hydroxyl group on vitamin E chromanol ring reacts with the peroxy radical to form the corresponding lipid hydroperoxide and tocopheroxyl radical. Thus, vitamin E acts as a chain-breaking antioxidant, preventing further lipid autoxidation, but it also forms a radical, which may re-initiate lipid peroxidation. Studies have shown that due to the synergistic interaction of antioxidants, combining vitamin E with vitamin E recycling antioxidants such as vitamin C, glutathione, ubiquinone, or carotenoids provide better antioxidant activity (Rozman and Gasperlin, 2007). There is growing evidence that vitamin E and C work together to enhance cellular function, despite being located in different parts of the cell. Through the regeneration of vitamin E radical, vitamin C increases the protective effectiveness of vitamin E. Therefore during UV exposure, vitamin E scavenges active free radicals and is oxidised to produce vitamin E radicals and in the presence of vitamin C, vitamin E radicals get reduced back to Vitamin E and thus both works synergistically. Thus, antioxidant capability of vitamin E is effectively increased by the synergistic interaction of vitamin E and vitamin C and thereby provide enhanced photoprotection to the cells. (Lin *et al.*, 2003) .

1.5 Hypothesis

A new formulation of GelMA based bioink that contain a cocktail of free radical scavenging (FRS) molecules will have the photoprotective property to ensure viability and functionality of 3D bioprinted liver construct.

1.6 Aims and objectives

The aim of the study is to develop GelMA based bioink formulation with photoprotective property for the three-dimensional bioprinting of liver construct.

To accomplish this aim, four objectives are formulated as given below.

1. Synthesis and characterization of Gelatin based formulations of bioink
2. *In vitro* evaluation of GelMA and printability for 3D bioprinting
3. *In vivo* biocompatibility studies of 3D printed constructs
4. Efficacy evaluation of cell laden construct

1.7 Scope of the study

The study will explore the synergic mechanism of antioxidant Vitamin C and Vitamin E to formulate a novel photo-protective GelMA based bioink for the fabrication of bioprinted liver construct.

1.8 Structure of thesis

The thesis is divided into five chapters Chapter 1-Introduction, Chapter 2-Literature review, Chapter 3-Materials and Methods, Chapter 4-Results and Discussion and Chapter 5-Summary and Conclusion. Chapter 1 introduces the area

of research and defines the research problem. To address the research problem a research hypothesis is formulated and the objectives through which the hypothesis can be proved are enumerated in this chapter. The chapter ends with a mention on the design and scope of the thesis. Chapter 2 reviews the relevant literature on the various approaches in liver tissue engineering mainly focusing on three-dimensional bioprinting. The evaluation of bioinks was also described. Chapter 3 contains a structured experimental design elaborating the materials, equipment and protocols involved in the study. Chapter 4 presents the obtained results related to each objective of the thesis and discusses the results in comparison to previous findings. Chapter 5 summarizes the results and draws a conclusion based on the major achievements and limitations of the study. The chapter ends with a remark on future direction of research related to this study.

Chapter 2

LITERATURE REVIEW

2.1 Liver tissue engineering

More than 500 million individuals worldwide suffer from chronic liver diseases, comprising 2% of total fatalities (Mazza *et al.*, 2018). According to the systematic analysis of the Global Burden of Disease Study 2017, 1.32 million people died in 2017 from cirrhosis and other chronic liver diseases, compared to 889 000 in 1990 (Fedeli *et al.*, 2023). Thus, the incidence of fatal liver diseases is increasing. Given this urgent situation, it is crucial to comprehend the physiological function of liver cells and the underlying disease mechanism to ensure effective treatment. Liver transplantation is currently the efficacious treatment for end-stage liver disease; however, there exists a significant disparity between the demand for organ transplantation and the availability of donor organs. The demand for donor organs continues to rise, therefore it is imperative to explore new therapeutic alternatives to liver transplantation. One such alternative is cell therapy, which involves administering live, whole cells to patients for disease treatment (Tatsumi and Okano, 2017). A potential cell therapy is allogeneic hepatocyte transplantation (HTx) where isolated human hepatocytes are infused and then engrafted into the recipient's liver to provide the impaired hepatic function. The source of human hepatocytes are mainly donor livers unsuitable for OLT or from liver segments mainly I and IV available after split liver transplantation. Even though HTx has shown encouraging clinical improvements

in patients, but the availability of donor livers from which high-quality hepatocytes can be isolated is limited. The cells isolated from liver rejected for OLT generally exhibit poor performance. Also, the hepatocytes for transplantation are stored under reduced temperature and the use of cryopreserved cells have limitation of reduced cell viability due to freeze thaw cycle (Iansante *et al.*, 2018). The long-term efficacy of engrafted cells is also limited by immunosuppression (Bhatia *et al.*, 2014). Therefore, to overcome the drawbacks of current cell-therapy approach, the development of tissue engineered implantable hepatic tissues is a promising strategy for the treatment of liver diseases.

Tissue engineering is an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function (Siemionow and Eisenmann-Klein, 2010). Tissue engineering has been identified as a promising strategy for achieving two main objectives: generating tissue and organ substitutes for clinical transplantation and creating human tissue analogues for drug testing and disease modelling (Mandrycky *et al.*, 2017). The three main components involved in tissue engineering are (1) cells (2) a suitable scaffold for support; and (3) bioactive molecules, such as cytokines and growth factors, that will encourage and coordinate the formation of the desired tissue (Krishani *et al.*, 2023). Liver tissue engineering (LTE) enables us to reproduce and restore liver functions, fully or partially, which could be used in the treatment of acute or chronic liver disorders and/or generate an appropriate functional organ which can be transplanted or employed as an extracorporeal device (Heydari *et al.*, 2020a). In LTE, hepatic models are mainly developed by combining primary or stem cell-derived cells with

three-dimensional (3D) scaffolds in an effort to mimic the complexity of tissue architecture. The successful development of cell microenvironment resembling the dynamic native extracellular matrix have increased the likelihood of being able to meet clinical demand for tissue (or organ) repair and replacement in a timely manner (Mazza *et al.*, 2018). *In vitro* biofabricated liver tissues can perfectly replicate the cell-to-cell and cell-to-matrix contact environment found *in vivo*, which aids in restoring liver cell polarity and enhancing growth activity. Also, in comparison to two-dimensional monolayer tissue, *in vitro* biofabricated liver tissue can maintain cell polarity for a long time and ensure cell surface protein transmission, preventing liver tissue function loss (Yang *et al.*, 2022). An adequate cell source, a 3D microenvironment with efficient mass transfer to maintain liver functions, and ease of handling and immobilization during transplantation are all necessary to biofabricate a hepatic construct *in vitro* for implantation (Yang *et al.*, 2021). Common TE techniques used for fabricating 3D hepatic model are based on distributing primary hepatocytes or hepatocyte-like cells derived from stem cells within a three-dimensional structure, thereby improving their survival rate and functional phenotype (Heydari *et al.*, 2020a).

2.1.1 Cell source

The selection of cells is critical in the process of biofabrication of liver tissues. In liver, hepatocytes form the functional metabolic unit, which accounts for 60–65% of the total liver's cell population. Along with hepatocytes, the non-parenchymal cells such as Hepatic sinusoidal endothelial cells (HSEC), Kupffer cells (KC), hepatic stellate cells (HSC), cholangiocytes, portal fibroblasts (PF) and

pit cells are also used for engineering of liver tissue (Mazza *et al.*, 2018). Finding patient-derived hepatocytes for tissue engineering applications is challenging, so various other sources of hepatocytes have been investigated. Adult hepatocytes, hepatoblasts, oval cells, foetal hepatocytes, hepatocytes derived from adult stem cells, and hepatocytes derived from induced pluripotent stem cells (iPSC) are some of the popular alternative sources of hepatocytes (Fitzpatrick *et al.*, 2009). The isolation and maintenance of hepatocytes and non-parenchymal cells *in vitro* is one of the major hurdles in liver tissue engineering. The 2D-matrix environment that the cells are exposed to in traditional culture conditions causes significant changes in the cellular phenotype over a brief period of culture.

2.1.2 Single cell type

2.1.2.1 Primary Human Hepatocytes

Primary human hepatocyte (PHH) is still regarded as excellent source for liver tissue engineering, including modelling infectious liver diseases, assessing drug metabolism, and liver regeneration. Hepatocytes accounts for 60-80% of the liver mass and are responsible for many important functions of the liver. Also, when there is liver damage *in vivo*, adult hepatocytes proliferate and aid in the regeneration of the liver. However, the applicability of PHH for tissue engineering is limited by its lack of proliferative capacity and rapid loss of functions during conventional *in vitro* culture (Hu *et al.*, 2015). Therefore, to overcome this limitation, various techniques, such as culturing in the form of spheroids, redefining the culture medium with growth factors, or culturing in a 3D system

mimicking the tissue microenvironment, have been developed and have shown improved functionality and viability *in vitro*. (Wang *et al.*, 2022).

2.1.2.2 *Animal primary hepatocytes*

Rat and porcine hepatocytes have been used in studying liver regenerative medicine. The main advantage of using animal cells is their availability. Rat hepatocytes are mainly isolated from Sprague Dawley and Wistar rats. Rat hepatocytes can be used as a source for preliminary studies. It is difficult to isolate rat hepatocytes as well as only a limited number of cells are obtained after isolation.

Pigs show similar physiology with human and exhibit functions closer to human liver tissue (Mirdamadi *et al.*, 2020). Therefore, porcine hepatocytes are used in Bioartificial Liver (BAL), a device that provides *in vivo* hepatic function in patients with liver failure. BAL requires large amount of functional cells and thus pig is considered as a best donor species (Donato *et al.*, 1999). The main limitations in using porcine hepatocytes are the transmission of zoonotic diseases, protein–protein incompatibility and the possibility of host immune reaction (Palakkan *et al.*, 2013).

2.1.2.3 *Induced Pluripotent Stem Cells*

Induced pluripotent stem cells (iPSC), are a type of pluripotent stem cell derived from adult somatic cells that have been genetically reprogrammed to an embryonic stem (ES) cell-like state that enables the development of any type of human cell (Ye *et al.*, 2013). Therefore, directing the differentiation of iPSC

towards hepatocytes have been explored as an alternative cell source to overcome the challenge of hepatocyte culturing. This method allows for the production of large numbers of hepatocytes for tissue engineering. iPSCs can be isolated from patient's own cells and thus can prevent any risk of immune rejection (Zakrzewski *et al.*, 2019). Many studies have reported on the development process for human iPSC differentiation into the hepatocytes. A study that evaluated the functions of differentiated hepatocytes from stem cells found that they could be used in cellular therapies. But the cells that are produced are not functionally mature when compared to *in vivo* conditions (Raju *et al.*, 2018). It is impossible to maintain hepatocytes made from embryonic or induced pluripotent stem cells as they do not proliferate. In addition, it has been demonstrated that iPSC reprogramming and cell epigenetic memory may follow different differentiation pathways due to phenotypic differences (Zhou *et al.*, 2012). The use of pluripotent stem cells has also given rise to concerns about the possibility of tumor development, the efficacy of long-term differentiation, and low differentiation efficiency (Ye *et al.*, 2013). Therefore, it is essential to create functional cells that are just as effective as primary cells.

2.1.2.4 Human Adult Liver Stem Cells

A mature liver contains a small percentage of hepatic progenitor cells, which can differentiate into liver cells. These adult stem cells are known as oval cells. They are similar to embryonic hepatoblasts, which are liver progenitor cells that can differentiate into cholangiocytes and hepatocytes, in terms of phenotype markers and functions (Zhang *et al.*, 2003). The tiniest branches in bile canaliculi

contain these cells. Isolated hepatic liver stem cells have the capacity to differentiate into bone, endothelial, insulin-producing, and hepatocyte cells. Studies have reported that HGF and FGF-4 enhance their differentiation to hepatocytes. Through a similar regeneration process showed by liver, these oval cells can partially repair the liver with acute and chronic liver diseases. The complicated isolation conditions of these cells, however, limit their application (Yang *et al.*, 2022).

2.1.3 Co-cultured Cell System

Liver is composed of hepatocytes and other non-parenchymal cells (NPC). The function of hepatocytes is correlated with hepatocyte–hepatocyte, hepatocyte–ECM and hepatocyte–NPCs interaction. In conventional culture systems, the absence of reciprocal interactions causes hepatocytes to rapidly lose both their phenotype and their functions (Tegge *et al.*, 2018). Therefore, to overcome this limitation, researchers proposed co-cultured systems where two different cell types are cultured together and are allowed to interact within the culture. Coculturing PHHs with other nonparenchymal cells (NPCs), such as sinusoidal endothelial and stellate cells, and fibroblasts to mimic their microenvironment, has also shown promising results. Similarly, coculture with hepatic stellate cells (HSCs) can simulate the matrix environment and vascular endothelial cells (VECs) can initiate angiogenesis and provide nutrition. Studies have also shown that combining hepatocytes with bone or adipose mesenchymal stem cells (MSCs), also supported liver function via paracrine cytokine secretion. Lee *et al.*'s study showed that HepG2 cells and MSCs when cocultured to examine the cells potential

for invasion, they discovered that HepG2 cells had high levels of markers for increased stemness, proliferative ability, and epithelial mesenchymal transition (EMT) (Lee *et al.*, 2021b). It has been reported that coculturing hepatocytes, fibroblasts, and VECs simulate liver lobule structure and significantly improved VEC capillary formation and maintained relevant liver function parameters (Liu *et al.*, 2014).

2.1.4 Hepatic spheroids and organoids

Spheroids are cell aggregates, formed by self-aggregation of single or multiple cell types when cultured in low adhesion environment. The spheroid formation mechanism is a complex process that involves cell-cell and cell-matrix interactions. Initially, the cells in suspension or on a substrate self-assemble through weak physical interactions and form cell-aggregates. These cell aggregates are then compacted by contracting actin-myosin filaments, resulting in mechanical forces and increased cell-cell adhesion. Spheroid culture helps to overcome the limitation of traditional monolayer cell culture and enhance the connections between cell-cell and cell-matrix (Białkowska *et al.*, 2020).

Several methods have emerged during the last few decades with the intention to produce spheroids in a high-throughput manner. Under specific culture conditions, such as an environment with extremely low adhesion, the micro molding technique, rotary culture, or the hanging drop method, spheroids can be produced. Spheroid cell culture provides cells a three-dimensional environment mimicking natural tissues. Spheroidal cells improve gene expression linked to apoptosis, survival, and angiogenesis while assisting in maintaining cell-to-cell

contact and intercellular signals. Spheroids have been utilized in tissue engineering, cancer research, and studies on drug metabolism because of these qualities (Cox *et al.*, 2020). The formation of hepatocyte spheroids (HSs), which were first described by Landry *et al.*, involved the self-aggregation of a single rat hepatocyte (Landry *et al.*, 1985). HSs have been reported to be transplantable in animal models. It has been established that HSs are capable of maintaining liver-specific functions because their structure closely resembles the environment of the original liver tissue. HSs were found to have higher viability and albumin production than typical cultured single hepatocytes. Furthermore, HS's spheroidal structure shields hepatocytes from apoptosis. These traits imply that HSs can lessen cell deterioration brought on by cell isolation and may enhance engraftment and/or survival following HCTx (Shibuya *et al.*, 2021).

The term “organoid” refers to a complex tissue-like structure with multiple cell types that functions as a “miniorgan” *in vitro* and spheroids are frequently described as “simple cell aggregates” made up of only one type of cell. There is growing evidence that 3D cell culture can promote a more *in vivo*-like phenotype than 2D cell culture. Studies have also shown that hepatocytes exhibit increased viability and functional capability when grown in sandwich cultures or self-assembled spheroids, demonstrating the clear benefits of three-dimensional growth conditions. The ability of liver spheroids and organoids to grow cells as 3D structures allows for the development of cell-cell interactions that are similar to those found in the liver's *in vivo* environment (Sato *et al.*, 2021). The hepatocyte spheroids show long term and stable liver-specific functions of hepatocytes. *In vitro* tests showed that the multidrug resistance-related protein-2 (MRP2),

albumin, and CD31 expression levels were higher in the structured microtissue spheroids. Researchers have created culture platforms that can generate large number of uniformly sized liver spheroid cells from iPSCs (Lee *et al.*, 2021a). Another 3D spheroid model is the liver bud (LB), which develops from a variety of pluripotent stem cells through interactions with the extracellular matrix to form tiny organs that resemble embryos (Wang *et al.*, 2006).

2.1.5 Scaffolds for liver tissue engineering

Cells, scaffolds and growth-stimulating signals are the key components in tissue engineering generally referred to as the tissue engineering triad. A scaffold is the structural framework made up of biomaterials for providing support to the cells to adhere, migrate, and subsequent development of functional tissues (Tomlins, 2016). A biomaterial is defined as “any substance, whether natural or synthetic, that can be used in the body to treat, augment, or replace tissues, organs, or bodily functions” (Gilmore *et al.*, 2014). In liver, the ECM component plays an important role in maintenance of cellular characteristics and regulation of functions. Therefore, biomaterial should be selected based on their ability to reproduce the essential physiological characteristics of ECM. Hydrogels, which are networks of organic or synthetic hydrophilic polymer chains, were among the first biomaterials intended for use in the human body (Taaca *et al.*, 2022). The liver ECM can be functionally mimicked by using hydrogels. They are among the most promising candidates for use as biomaterial in liver tissue engineering (Pal *et al.*, 2012). Scaffolds can be based on either natural or synthetic biomaterials. Natural biomaterials are derived from animal bodies, plants or marine world which

include cellulose, chitosan, gelatin, alginate, collagen, fibrin, heparin, hyaluronic acid, etc. Additionally, tissue-derived materials, which are primarily decellularized ECM obtained by using physical, chemical, and enzymatic agents removing all cells from native tissues or organs, are also been widely studied (Brovold *et al.*, 2018). Some of the synthetic biomaterials used are poly(vinyl alcohol), poly(L-lactic acid), poly(N-isopropylacrylamide), poly(lactide-co-glycolide) and polycaprolactone or modified polymers.

During *in vitro* culturing of cells in a scaffold, it is necessary to use scaffolds capable of recreating the *in vivo* microenvironment in such a way that they provide biophysical, biomechanical, and biochemical cues that maintains cell viability, proliferation, differentiation and function. The scaffolds also have to be porous enough to facilitate nutrient diffusion and oxygen transfer for the survival of cells (Sultana, 2018). During *in vivo* applications, the scaffold should also exhibit biodegradability as well as biocompatibility. Biocompatibility can be defined as “the ability of an implant material to function *in vivo* without eliciting detrimental local or systemic responses in the body”(Ratner, 2011). Therefore, upon implantation, the scaffolds should degrade either spontaneously or by the action of biological enzymes without producing toxic byproducts and eliciting host-immune reaction (Sengupta *et al.*, 2014). The degradation rate must be equal to or slower than the rate of new tissue formation to ensure appropriate tissue healing.

Natural biomaterials often contain specific protein-binding sites and thus encourages cell adhesion, migration, proliferation, and differentiation. They also show excellent biocompatibility, biodegradability, and remodeling capacity

(Joyce *et al.*, 2021). Currently, liver tissue engineering makes extensive use of natural biomaterials. The mechanical properties of the scaffolds are also important in LTE as they offer structural support to create the ideal environment for cell loading and tissue development, ultimately promoting liver regeneration. The mechanical properties of natural polymers are often constrained. So, natural polymers mixed with other natural or synthetic polymers are numerous and have also been studied for the generation of appropriate tissue engineering scaffolds.

2.2 Approaches in LTE for clinical application

The presence of functional hepatocytes with effective nutrient transport and secretion of essential hepatic factors, such as albumin and coagulation factors, within the engineered hepatic tissue is a critical requirement for the development of a therapeutic product for the treatment of liver failure. It is necessary for the transplanted engineered tissue to survive for a long time inside the host. Diverse methodologies can be employed to generate engineered hepatic tissues such as cell encapsulation, cell sheet, microfluidic, bio assembly, 3D bioprinting (Mazza *et al.*, 2018).

2.2.1 Cell encapsulation

Encapsulation method is the embedding of cells in a semipermeable polymerized structure, which aims to shield them from host immune attack while allowing the diffusion of nutrients, oxygen, and metabolic products that enables cell function and survival (Heydari *et al.*, 2020a). Encapsulated hepatocyte transplantation and encapsulated mesenchymal stem cell transplantation are both

new potential treatments for acute and chronic liver diseases. Researchers found that intraperitoneal transplantation of rat hepatocytes in an alginate capsule could provide enough metabolic support to save animal models of acute liver failure for up to 7 days without immunosuppression (Jitraruch *et al.*, 2014). Even in small infant's intraperitoneal infusion of micro-encapsulated cryopreserved human hepatocytes in ultrapure GMP grade alginate was found to be feasible and safe in children with acute liver failure (Dhawan *et al.*, 2020). There are studies that reports human stellate cells co-cultured with human induced pluripotent stem cell-derived hepatocyte-like cells (iPSC-HLC) and enclosed in alginate beads remained in immunocompetent mice for 24 days without any immune rejection. Despite the fact that alginate is biocompatible, biodegradable, and non-toxic it has a number of drawbacks, including low bio adhesivity and biological inertness, which restrict its applications in tissue engineering. Alginate is obtained from brown seaweed or bacteria so it is challenging to separate pure alginate from tainted marine algae and so additional purification steps are necessary to remove impurities, and they are costly and time-consuming (Labowska *et al.*, 2021).

In another study, chitosan/silk fibroin hydrogels, crosslinked with genipin was evaluated for the development of 3D vehicle for the delivery of hepatocytes in cell therapy (Gholami *et al.*, 2023). However, more studies are required to advance the cell encapsulation method, including more research in various liver disease models, a decrease and modification of the fibrogenesis response during inflammation, and an enhancement of neovascularization through model structure.

2.2.2 Cell sheet engineering

Cell sheet engineering is an advanced scaffold-free cell-based tissue engineering technique which is used to generate transplantable single or multi-layered cell-dense sheets to achieve tissue repair and regeneration (PATIL *et al.*, 2024). In cell sheet-based tissue engineering, cell sheets are separated from temperature-responsive culture dishes. In thermosensitive culture dishes, temperature sensitive polymers are covalently grafted on the surface of culture dishes and when the culture temperature is lowered, cultured cells are detached from the surface and harvested as an intact monolayer which can directly applied for therapeutic use. This technology allows us to prepare transplantable tissue constructs while preserving intact cell-cell junctions and extracellular matrices without the need for enzymatic digestion of the cells (Tatsumi and Okano, 2017). A study showed that mice with acute liver damage from carbon tetrachloride were successfully treated with the transplant of hepatocyte-like cells derived from induced pluripotent stem cells (iPS-HLC) sheets onto their liver surfaces (Nagamoto *et al.*, 2016). Sakai *et al.*, have successfully achieved therapeutic effects in animal models by performing hepatocyte sheet transplantation into ectopic sites (such as the subcutaneous space) and suggests the use of subcutaneous hepatocyte sheets for the treatment of different liver conditions as a desirable option (Sakai *et al.*, 2015). Orthotopic transplantation of hepatic cell sheets created from differentiated human mesenchymal stems was shown to be therapeutically effective on mice with acute liver injury. The rate of cell survival was significantly increased after the cell sheets were transplanted, which improved liver regeneration and reduced liver damage (Itaba *et al.*, 2019).

Although the majority of the current findings come from studies using rodent models, attempts using large animal models and subsequent clinical trials are necessary to determine whether transplantation of cell sheets made of hepatocytes or hepatocyte-like cells is feasible as a therapeutic approach for treating liver diseases.

2.2.3 Decellularization-recellularization method

The engineering of whole organs has been impeded by the lack of an adequate scaffold. A strategy for whole organ tissue engineering that has gained popularity recently is decellularization-recellularization method. Decellularization is the process by which cells are removed from tissues/organs, but all of the essential cues for cell preservation and homeostasis are retained in a three-dimensional structure of the organ (Fu *et al.*, 2014). Thus, retaining extracellular matrices of structural and functional proteins that are unique to that organ offer essential microenvironments that promote organ regeneration. Decellularized ECMs also maintain the native organs original vascular network structures which allows the tissue to be connected directly to the patient's vasculature during transplantation, allowing for immediate circulation and delivery of nutrients and oxygen (Mazza *et al.*, 2018). The most dependable technique for obtaining decellularized whole-organ scaffolds for use as structures in organ bioengineering is perfusion-decellularization (Guyette *et al.*, 2014). Decellularized ECM-derived scaffolds have been used by researchers to make transplantable organ grafts for liver, lung, kidney, and heart. For the decellularized scaffold to perform functions, they need to be repopulated with functional cells. During recellularization, after

seeding cells into the decellularized liver matrix, the neo-organ matures in the bioreactor, and bioengineered graft can be implanted without the need for lifelong immunosuppressive therapy. Studies shows that recellularized liver graft exhibited liver specific functions compared to normal liver *in vitro*. Also, it was demonstrated that recellularized liver when implanted into rat liver showed hepatocyte survival and minimal ischemic damage (Uygun *et al.*, 2010). In another study, a humanized porcine liver was developed using decellularized porcine liver and co-cultured human foetal hepatocytes, and human foetal stellate cells. Immunohistochemical analysis demonstrated the differentiation of cells into mature hepatocytes. Also, it was observed that 40% of the transplanted cells were undergoing active proliferation, while a minority of less than 30% exhibited signs of apoptosis (Barakat *et al.*, 2012). Using large animal model with liver dysfunction, a recent study showed the long-term utility of decellularized liver scaffolds for regenerative therapy. This was the first study showing the clinical value of this novel strategy by examining the functions of engineered liver grafts post-transplant for 28 days in a preclinical large animal model (Higashi *et al.*, 2022). However, more research is required to confirm the technique's clinically relevant safety and efficacy before it can be used as a substitute for organ donation and to lessen the burden of ongoing immunosuppressive therapy.

2.2.4 Bioassembly

In traditional tissue engineering method, the cells are seeded on already fabricated biomimetic scaffolds and it results in a heterogeneous tissue with an uneven cell distribution. So, it is necessary to produce constructs that more

accurately mimic the complexity and heterogeneity of tissues and organs. Biofabrication is “*the automated generation of structurally organized, biologically functional products from living cells, bioactive molecules, biomaterials, cell aggregates such as microtissues or hybrid cell- material constructs through bioprinting or bioassembly, and subsequent tissue maturation processes*” (Groll *et al.*, 2018). Bioassembly enables the automated assembly of cell-containing building blocks. The ability of cells to self-assemble is required for directed tissue self-assembly. Spheroids are frequently used as building blocks for the biofabrication of living tissues where spheroids when placed close together, they self-fuse in an almost liquid-like manner. As a result, the use of spheroids for self-assembling and self-organized tissue fabrication appears promising. A scaffold-free approach can avoid several issues that are connected to the use of exogenous materials in scaffold-based tissue engineering approaches, such as infection, immune reactions, material deterioration, and biofilm formation. According to reports, using the acoustic bioassembly technique, researchers constructed a scaffold-free hexagonal cytoarchitectures *in vitro* to mimic the cord and sinusoid structures found in hepatic lobules using endothelial cells and liver spheroids made from human induced pluripotent stem cells and it has the potential to be widely used in tissue engineering and regenerative medicine (Gu *et al.*, 2022). In another study, researchers used a needle array method to fuse hundreds of liver bud-like spheroids and created scaffold-free liver-like tissue which was then transplanted on rat liver and the engraftment of biofabricated tissue was evaluated. The findings support the notion that orthotopic transplantation of biofabricated tissue creates an environment that is favorable for liver tissue

engraftment and demonstrate a novel method of cell-based therapy that may result in liver tissue transplantation (Yanagi *et al.*, 2017).

2.2.5 3D Bioprinting

3D bioprinting is a promising technology that allows for highly precise and controlled fabrication of functional living tissue *in vitro* by controlling the deposition of cells and biomaterials (Lu *et al.*, 2021). In 3D Bioprinting process, it combines 3D printing technology, cell biology and material science to create tissue constructs resembling native tissues. Charles W. Hull first described the field of 3D printing as “stereolithography” (STL) in 1986. In this method, solid 3D structures were created using photochemical processes by layering thin layers of a material. Since 1990s, the scaffolds for tissue engineering have been prepared with sacrificial resin molds by STL. With the advancement in 3D printing technology and material science, 3D bioprinting was emerged as a technique for fabricating functional tissues. When compared with conventional scaffold based TE approaches where cells are seed on prefabricated scaffolds, 3D bioprinting is better to mimic the complex microstructures of biological tissues with precise distribution of cells (Zhang *et al.*, 2021). Furthermore, it gives the opportunity to achieve high initial cell densities and distribute various cell types at desired locations within the bioprinted construct (Kryou *et al.*, 2019).

The three distinct steps of three-dimensional bioprinting process are preprocessing, processing, and postprocessing (Figure 5). In preprocessing phase, a 3D model of the tissue or organ is designed using computer aided designing software (CAD) based on the medical images of the damaged tissue or organ. The

processing phase includes the development of an ideal bioink formulation and effective bioprinting of tissues with desired properties. Finally, in the postprocessing phase, after *in vitro* tissue maturation, the bioprinted constructs will be characterized and used for further applications (Pai *et al.*, 2022b).

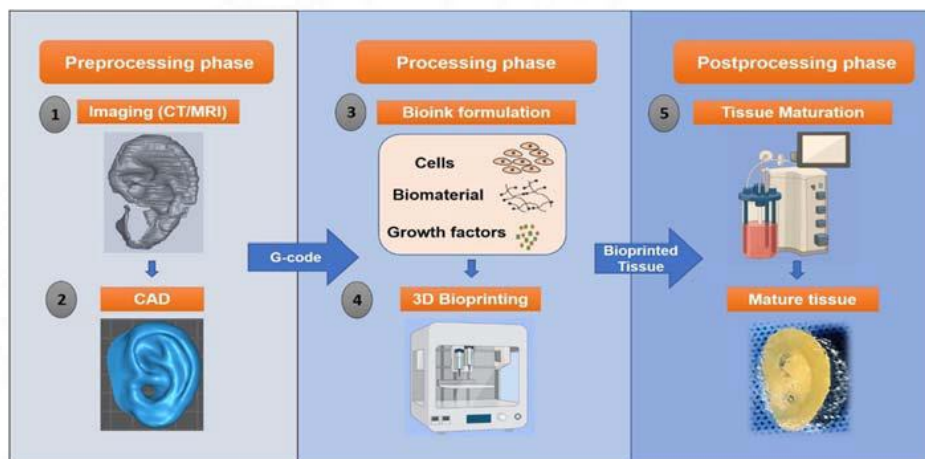


Figure 5. Schematic of 3D bioprinting workflow. Adapted from (Pai *et al.*, 2022b)

There are different bioprinting technologies based on the working principle, and the main bioprinting techniques are extrusion, inkjet, and laser-induced forward transfer (LIFT) is shown in Figure 6. Studies have shown that extrusion based bioprinting is the predominant technique for the bioprinting of liver models (Ali *et al.*, 2024). With the advent of bioprinting, the creation of functional tissue livers has become a near-certainty (Kryou *et al.*, 2019). Three-dimensional hepatic cell culture, which can produce complex cell structures, shapes, and arrangements, can greatly improve experiment precision and reliability.

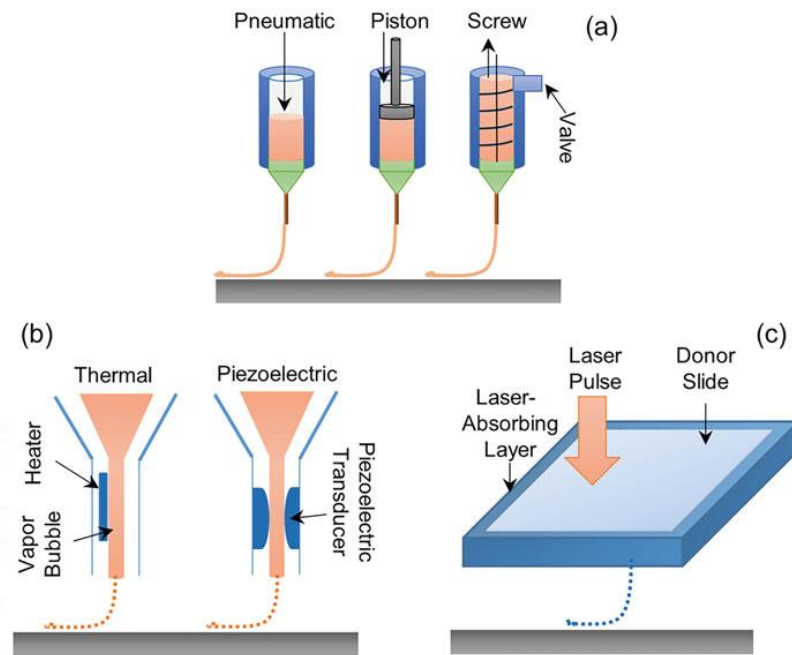


Figure 6. Different types of bioprinting technologies. (a) Extrusion based bioprinting, (b) Inkjet bioprinting and (c) Laser-assisted bioprinting. Adapted from (Anupama Sekar *et al.*, 2021)

There have been numerous studies reported on the fabrication of bioprinted liver tissues with efficient *in vitro* and *in vivo* functions. Nguyen *et al.*, have shown that a 3D bioprinted human liver tissue generated using extrusion bioprinter was stable and exhibited liver specific functions for 28 days. The study also demonstrated a multicellular liver structure that included hepatocytes, hepatic stellates, and endothelial cells (Deborah G. Nguyen^{1*}, 2016). In a study conducted by Bhise *et al.*, a bioprinted liver tissue with improved liver-specific functionalities and gene expression, as well as drug response comparable to *in vivo* animal studies were reported (Bhise *et al.*, 2016). The studies on efficacy evaluation of bioprinted liver after transplantation into animal models also demonstrated that transplanted group had improved liver function compared to the control group and had a longer survival time, indicating the efficiency of 3D

bioprinting for liver function reconstruction and liver tissue regeneration (Zhong *et al.*, 2016, Yang *et al.*, 2021). Therefore, 3D Bioprinting technology provides a strong biofabrication platform for generating complex and functional liver tissues.

2.2.6 Bioinks

Bioink is one the main element in the 3D bioprinting process that plays a significant role in the successful bioprinting of tissues. Aqueous formulations of polymers or hydrogel precursors that contain cells and biological factors constitutes a bioink. In this regard Groll *et al.*, defined bioink as “a formulation of cells suitable for processing by an automated biofabrication technology that may also contain biologically active components and biomaterials” (Groll *et al.*, 2018). Therefore, in a bioink formulation, cells are the major constituent and the biomaterial provides physical and chemical cues for efficient functioning of the bioprinted tissue. Thus, the selection of an appropriate bioink with certain properties is essential for the biofabrication of functional tissues by bioprinting. Among the different forms of biomaterials, hydrogels are considered as the most suitable biomaterial for bioink formulation.

Hydrogels are three-dimensional network of crosslinked hydrophilic polymers. It can absorb and hold large amount of water and thereby they are capable of mimicking the native extracellular matrix environment. Hydrogels can be natural or synthetic polymers based on origin. In liver, it is composed of protein and polysaccharide-based ECM, so natural hydrogels mimicking the ECM components can have a significant impact on promoting liver cell growth *in vitro*

and are better suitable for reestablishing the cellular microenvironment *in vivo* (Lv *et al.*, 2022).

Table 1: Bioinks used in the 3D bioprinting of liver tissues

Cells	Biomaterial	References
HepG2/C3A	GelMA	(Bhise <i>et al.</i> , 2016)
hiPSC, hematopoietic progenitor cells (HPCs), HUVEC, and adipose-derived stem cells	GelMA	(Ma <i>et al.</i> , 2016)
Human adipose-derived stem cells (hASCs)	Collagen	(Lee <i>et al.</i> , 2017)
HepG2	Alginate	(Jeon <i>et al.</i> , 2017)
HUVECs and HepG2	Glycidal methacrylate-hyaluronic acid (GM-HA)	(Zhu <i>et al.</i> , 2017)
HepaRG and human HSCs	Gelatin + PEG	(Grix <i>et al.</i> , 2018)
HepG2	Liver dECM + GelMA	(Ma <i>et al.</i> , 2018)
HepG2 and NIH/3T3 fibroblast	GelMA	(Cui <i>et al.</i> , 2019)

Natural polymers include collagen, alginate, gelatin, cellulose, hyaluronic acid, fibrin and so on. Since decellularized ECM retains the intrinsic cues of a native tissue, it is also considered as an excellent source of bioink. Studies on synthetic polymers (polyethylene glycol (PEG), Pluronic) are also been reported. However, natural polymers exhibit poor mechanical strength and synthetic polymers lack native ECM characteristics, so natural polymers are modified with chemical moieties (e.g. GelMA) and synthetic polymers are functionalized with bioactive sites (e.g. PEG-RGD). Bioinks composed of multicomponent (two or more biomaterials) are also frequently studied to address the limitation in physical and biological performance of individual biomaterials (Kim *et al.*, 2022).

2.3 Evaluation of bioinks

The formulation of a bioink is a crucial step in 3D bioprinting and the formulated bioink must be evaluated to ensure that it meets the necessary requirements. The properties of bioink can affect the stability and structural integrity of the final construct. The maintenance of cell survival during pre-printing, printing and post-printing process should also be ensured by the properties of the bioink (Jose *et al.*, 2024). Therefore, an ideal bioink formulation should possess certain physiochemical and biological properties, such as printability, mechanical properties, biocompatibility, biomimicry, and biodegradability for the successful application of a bioprinted construct (Ashammakhi *et al.*, 2019).

2.3.1 Printability

According to Murphy and Atala, printability is defined as “the properties that facilitate handling and deposition by the bioprinter” (Murphy and Atala, 2014). During 3D bioprinting, bioinks are precisely deposited in a layer-by-layer manner and printability of the bioink is the key component of this process. The rheological properties and crosslinking mechanism of the hydrogel along with the printing conditions are the factors that influences the bioink’s printability (Zhang *et al.*, 2018). In extrusion bioprinting, the printing conditions that can affect printability includes feed rate, pressure, path design, nozzle height, temperature etc. (Gillispie *et al.*, 2020) . In case of bioinks, before printing begins, they are in a resting state and then undergoes a transition to a high shear condition while passing through the nozzle, takes a new shape, and finally reaches a new resting

state and regain its stability after printing (Zhang *et al.*, 2018). In order to achieve this, a bioink should exhibit good thixotropic property and shear thinning behavior, which enable the bioink to decrease in viscosity when dispensed with a sufficient shear rate (Zhang *et al.*, 2018). These properties of bioink also help in reducing the shear stress exerted on cells in the bioink as they pass through the narrow orifice of the nozzle during bioprinting and thus protect the cells from damage during printing (Holzl *et al.*, 2016). The key rheological properties influencing the bioink transition during printing are viscosity, viscoelastic shear moduli, elastic recovery, and shear stress (Bom *et al.*, 2022). The analysis of rheological properties by rheometer is considered as a preliminary step in assessing printability and helps to determine a bioink's ability to print and also to optimize ideal printing conditions (e.g. printing temperature).

The basic requirement to achieve printability is extrusion. Therefore, printability is evaluated by analyzing filament extrusion, filament uniformity, filament shape, filament fusion, filament collapse and the ability to print sharp angles and maintain structural integrity (Schwab *et al.*, 2020). The ideal bioink formulation is expected to form continuous stable filaments rather than droplets during extrusion. After analyzing the deposition of filaments, it is necessary to evaluate shape fidelity of printed patterns. Shape fidelity refers to the shape retention of extruded single-layer filaments as well as the printed whole construct in such a way that final printed construct resembles the CAD model (Schwab *et al.*, 2020). Shape fidelity of the construct is also necessary to maintain the microchannel patterns designed within the constructs to facilitate oxygen transfer, nutrient diffusion, and the removal of metabolic waste (Gillispie *et al.*, 2020). In

order to achieve shape fidelity, deposited filaments should not collapse or fuse together and printed layers should stack without merging. The most commonly used mechanism to preserve the shape and structural integrity of the printed construct is through crosslinking of the bioink referred to as Hydrogel fixation (Holzl *et al.*, 2016) (Schwab *et al.*, 2020) .

2.3.2 Crosslinking mechanisms of hydrogel precursors

Depending on the nature of the polymeric backbone and functional groups present in the prepolymer, hydrogel bioinks can be crosslinked using various methods that can be chemical, physical, enzymatic or a combination of them. Physical crosslinking includes thermocondensation, self-assembly, ionic gelation, or electrostatic interaction. A common example for ionic crosslinking is the use of Ca^{2+} ions for crosslinking sodium alginate (Ashammakhi *et al.*, 2019). Hydrogels that have been physically crosslinked usually show poor mechanical stability. So, polymers have been modified with chemical functionalities to enhance the hydrogel mechanical strength by forming covalent crosslinks, leading to irreversible crosslinked network (Holzl *et al.*, 2016). Photo-crosslinking is a type of chemical crosslinking that is initiated by light (UV/Visible) in presence of photoinitiator and allows the formation of stable hydrogels. One of the widely used method for modifying polymers is by methacrylation. In natural polymers, they contain primary amino groups which are reacted with methacrylic anhydride (MAA) to add methacrylate group (MA) to the molecule. So, under the exposure of light and the presence of photoinitiator that absorbs light of specific wavelength initiates radical formation. These radicals then interact with the functional group

in the monomer and form covalent bond between the monomers and thereby polymerization occurs. Thus, methacrylate modification allows formation of stable crosslinked hydrogels through free-radical initiated polymerization (Moon *et al.*, 2023).

Gelatin, a collagen derivative is one of the widely studied biomaterial for hepatic bioprinting as it provides ECM like microenvironment. However, the lack of thermal stability at physiological temperature has limited its application in the development of bioink. Therefore, gelatin modified with methacrylate results in the formation of GelMA that can undergo photo-crosslinking under UV exposure in presence of photoinitiators like Irgacure 2959, LAP and form irreversible stable hydrogels (Yue *et al.*, 2015a). The schematic of the synthesis of GelMA and crosslinking is shown in Figure 7. In some cases, a two-step polymerization strategy is also preferred to obtain a stable bioprinted construct. It has been reported that by adding gelatin along with GelMA in bioink formulation, a two-step cross-linking combining the rapid and reversible thermo-crosslinking of gelatin with irreversible photo-crosslinking of GelMA has improved the shape fidelity of bioprinted construct (Yin *et al.*, 2018).

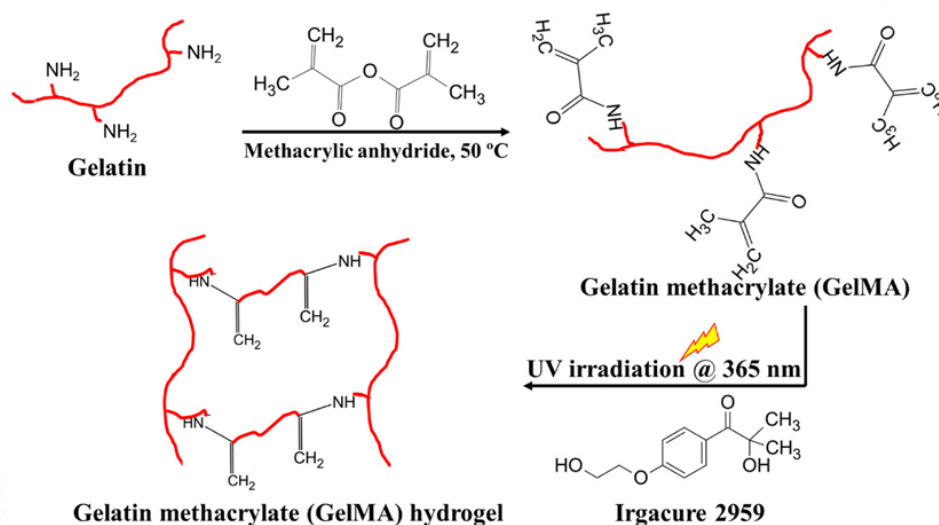


Figure 7. Synthesis and photo-crosslinking of GelMA under UV exposure in presence of photoinitiator. Reproduced with permission from (Zennifer *et al.*, 2022)

2.3.3 Biocompatibility

The biocompatibility of a bioink for 3D bioprinting can be referred to as the ability of bioink to effectively perform its intended function, with appropriate cell activities such as viability, adhesion, proliferation, and differentiation to aid tissue regeneration without eliciting any negative effect in the recipient host (Kim, 2023). Therefore, the bioink components has to be non-toxic, non-immunogenic, non-thrombogenic, non-carcinogenic, and non-mutagenic in order to exhibit an appropriate biological response (Parak *et al.*, 2019). Thus, the biocompatibility evaluation is a critical step in bioink development, as it determines the whether a bioink can be used for *in vivo* applications.

The biocompatibility of the bioink can be assessed with a series of standardized *in vitro* assays and then proceeding to a stringent set of standardized *in vivo* tests and finally with clinical trials by which the information about the

potential for adverse reactions, such as inflammation, infection, cellular responses to the matrices, and tissue damage can be obtained. The *in vitro* and *in vivo* response of the biomaterial can be evaluated based on the International Organization for Standards (ISO), Standard 10993, Biological Evaluation of Medical Devices. The ISO 10993 is the most extensive and standardized document available intended to provide international standards and methodologies to evaluate biomedical materials and devices. The ultimate goal of biocompatibility evaluation is to ensure the bioink is safe and effective for intended application (Kim, 2023).

2.3.3.1 *In vitro* evaluation

In addition to the printing conditions, the components of the bioink (concentration of polymers and photoinitiators) and the crosslinking mechanism utilized for stabilizing the printed construct can cause impact on the cell viability and thereby the functionality of the bioprinted construct. The hydrophobic nature of photoinitiators (PI) allows them to pass through the cell membrane and induce cytotoxicity. In a study conducted by Bryant *et al.* the viability of NIH/3T3 fibroblast cells in the presence of various photoinitiators, it was observed that among the three PIs studied, Irgacure 2959 exhibited least toxicity due to its relative polar nature when compared with Irgacure 184 and Irgacure 651 (Bryant *et al.*, 2000). The intrinsic toxicity of PI can be reduced by lowering its concentration; however, in order to produce hydrogels with good stability, either light intensity or UV exposure time has to be increased. But, increasing the light intensity or crosslinking time, could lead to the increase in the free radicals

produced during crosslinking which are potentially cytotoxic and mutagenic (Lee *et al.*, 2020). Therefore, to qualify any formulation for use as a bioink, it is essential to confirm its cytocompatibility.

Cytotoxicity and cell viability assays have been used as the screening method to verify the cytocompatibility of the biomaterial used in the bioink. The *in vitro* cytotoxicity of the material can be evaluated based on ISO10993: Part 5 and there are three primary *in vitro* assays; (1) the agar diffusion test, (2) the direct contact test and (3) the elution, or extract dilution assay (Kim, 2023). The changes in the cell morphology and viability followed by direct contact with crosslinked hydrogel or treatment with hydrogel extracts are assessed to determine the cytotoxicity of hydrogel.

The viability of the cells in the bioink after bioprinting and crosslinking can be qualitatively and quantitatively assessed using various assays commonly used for hydrogels such as alamar blue, lactate dehydrogenase (LDH), Trypan Blue (TB), and live/dead (FDA/PI, CaAM/EthD-1) assay. The adhesion, proliferation, differentiation of the cells when encapsulated in the bioink formulation also has to be evaluated for determining the biological properties of the hydrogel.

2.3.3.2 *In vivo* evaluation

An *in vivo* study involves testing within a living organism. The biocompatibility of the hydrogel used in the bioink can be evaluated by implantation in animal models. Based on ISO 10993: Part-6 which specifies test methods for the assessment of the local effects after implantation of biomaterials

intended for use in medical devices, the hydrogel can be assessed for its biocompatibility (J *et al.*, 2023). Implantation tests assess the local pathological effects on the tissue at the implantation site.

In a bioink evaluation, upon hydrogel implantation, an interaction between the host immune system and the implant occurs, leading to biomaterial-specific cellular and tissue responses. These responses may include inflammatory, wound healing responses, immunological and foreign-body reactions, and even fibrous encapsulation (Anderson *et al.*, 2008). In implantation study, the main aim is to evaluate the hydrogel integration into the host tissue, biodegradation and the resulting tissue response. The local effects at the implantation site are evaluated by comparing the tissue response at implant site of the test material to that of a control material with established clinical acceptability and biocompatibility characteristics (Sastri, 2014). The most basic evaluation of the local pathological effects is carried out at both the gross level and the microscopic level. The biological responses at the implantation site are assessed by histological evaluation. Therefore, it is necessary to formulate bioink with ideal properties for the successful bioprinting of tissue constructs and their subsequent success in applications.

Chapter 3

MATERIALS AND METHODS

3.1 Synthesis and characterization of Gelatin Methacrylamide

3.1.1 Gelatin Methacrylamide synthesis

Gelatin Methacrylamide (GelMA) was synthesized by functionalizing gelatin with methacrylic anhydride in carbonate-bicarbonate (CB) buffer, based on previous reports (Zhu *et al.*, 2019, Pai *et al.*, 2022a). The synthesis of GelMA involves three steps: (i) Functionalization, (ii) Purification and (iii) Drying.

The CB buffer (0.1M) was prepared by dissolving 1.59 g of Sodium carbonate and 2.93 g of Sodium bicarbonate in 500 mL of milliQ water. To synthesize GelMA, 20 g of Type A porcine skin gelatin with a bloom value of 175 (Merck-Sigma, Cat. No. G-2625, Germany) was dissolved in 200 mL of CB buffer (pH 10.5) at 60 °C with stirring on a hotplate with magnetic stirrer. The temperature was then lowered to 50 °C and 2 mL of methacrylic anhydride (MAA: Merck-Sigma, Cat. #276685, Germany) was sequentially added in a step wise manner to the dissolved gelatin while stirring at 300 rpm (i.e., 334 μ L at an interval of 30 min for 3 h). The pH of the reaction mixture was checked using pH paper before each MAA addition and if the pH<9, the pH was adjusted to 9 with CB buffer. At the end of the reaction, the pH of the GelMA solution was checked to ensure it is in the range of 7.4.

For purifying the synthesized GelMA solution and remove any unreacted MAA and short chain polymers, the resulting GelMA solution was transferred to dialysis tubing with a molecular weight cut-off of 12-14 kDa (Merck-Sigma, Cat. No. D9652, Germany). The GelMA solution was then dialyzed against water at 40 °C along with stirring at 150 rpm for 4 days with distilled water change twice every day with an interval of 6-8 h.

Finally, the dialyzed GelMA was freeze-dried continuously for 2 days in a lyophilizer (CHRIST, Alpha 1-2 LD plus, Germany) at -80 °C. The weight of the dried GelMA was measured and percentage yield was calculated. The lyophilized polymer (GelMA) was stored at room temperature until further use. The GelMA synthesis efficiency was determined by calculating the percentage yield achieved in various batches.

$$\text{Yield \%} = \frac{\text{Final dry wt of GelMA}}{\text{Initial dry wt of Gelatin}} \times 100 \dots\dots\dots \text{Equation 1}$$

3.1.2 Degree of functionalization

The degree of functionalization (DoF) was estimated by two assays: Ninhydrin and 2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA) method. Linear concentrations of gelatin 10, 8, 6, 4, 2% (w/v) was used as standard for Ninhydrin assay and concentration of 8, 16, 32, 64, 128 and 256 µg/mL Glycine was used to prepare standard for TNBSA assay.

In Ninhydrin assay, both gelatin and GelMA samples at a concentration of 10% (w/v) was prepared by dissolving in deionized water at 60°C. The gelatin solution was then diluted in water to prepare standard solutions of concentrations

(8%, 6%, 4%, and 2% w/v). Ninhydrin solution was prepared by dissolving 2.5 mg ninhydrin in a mixture of 0.5 M sodium citrate monobasic: glycerol at a 1: 2 ratios. 150 μ L of water (Blank), GelMA sample and Gelatin standard solutions were transferred to prelabelled 1.5mL tubes and to that 850 μ L of ninhydrin solution was added. The mixture was then incubated at 50 °C for 12 mins. After cooling to room temperature, 200 μ L of the solution from each tube was transferred to a 96-well plate. The absorbance of the sample and standards were measured at 570 nm using a multi-well plate spectrophotometer (Biotek, Model# Powerwave XS, USA). A linear regression line was drawn using the absorbance values obtained from standards. The concentration of unreacted groups in GelMA was calculated using the absorbance obtained for a gelatin concentration of X% in the standard curve. The DoF was calculated as 100–X %, considering that the reduction in absorbance was due to functionalization of free amino group by methacrylation.

In TNBSA assay, the degree of functionalization (DoF) was defined as the percentage of amino groups (from lysine and hydroxylysine) modified in GelMA. To calculate the amino group content in the GelMA samples, glycine standard curve was plotted using standard glycine solutions prepared at concentrations of 8, 16, 32, 64, 128 and 256 μ g/mL in 0.1M Sodium bicarbonate solution. Gelatin and GelMA samples at a concentration of 1.6 mg/mL was prepared by dissolving in 0.1M Sodium bicarbonate buffer. 125 μ L of glycine standards, gelatin and GelMA was transferred into labelled test tubes and 125 μ L of 0.1M Sodium bicarbonate alone was transferred into tube labelled as blank. Then 125 μ L of 0.01% TNBSA was added into each tube except in blank. The tubes were

incubated at 60 °C for 3h. After incubation, 62.5 µL of 1N Hydrochloric acid was added into each tube and only after addition of HCl, 125 µL of 0.01% TNBSA was added into blank tube. For absorbance measurements, 200 µL from each tube was transferred into a 96-well plate and absorbance was measured at 340 nm with a microwell plate reader (Biotek, USA). A linear regression line was drawn using the absorbance values obtained from glycine standards. The absorbance at 340 nm corresponds to the concentration of free amine groups; any reduction in free amine concentration is assumed to be due to methacrylamide functionalization. The absorbance of GelMA and gelatin corresponds to glycine concentration was calculated from the standard curve and the degree of functionalization was then calculated as (100-X) %.

3.1.3 Proton Nuclear Magnetic Resonance Spectroscopy (¹H NMR)

¹H NMR analysis was performed to confirm the functionalization of gelatin with MAA. 10 mg each of gelatin and GelMA was dissolved in 1 mL deuterium oxide (D₂O) by heating in a water bath at 40 °C. After complete dissolution, the polymer solutions were transferred into glass tubes. The NMR spectrum was obtained using a Bruker Avance 400 MHz FT-NMR spectrometer (Germany).

3.1.4 Photocrosslinkability of GelMA

Three different concentrations (3%, 4%, and 5% w/v) of GelMA hydrogel precursor solution were prepared by dissolving specific amount of lyophilized polymer in 1X PBS (0.01M, pH 7.4) (Takara, Cat# T9181) at a temperature of

37 °C. A photoinitiator solution of concentration of 5% w/v Irgacure-2959 (2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone) (Merck-Sigma, Germany) was prepared in PBS by heating in a water bath at 80 °C, and was added to the polymer solution to achieve a final concentration of 0.5% (w/v). The solution was thoroughly mixed by continuous pipetting, and approximately 200 μ L of each GelMA solution was transferred to a custom-made Teflon mold with cavities to cast samples of dimensions 10 x 2 mm. The hydrogel precursor solution was then exposed to UV light of 365 nm at intensity of 8–12 mW/cm². Finally, the crosslinked hydrogels were taken out and their morphology was examined.

To further investigate the photo-polymerization of 3, 4 and 5% GelMA formulations, the samples were exposed to a 365 nm UV light source Omnicure 1000 (Lumen Dynamix LDGI) at 20 °C under a constant shear strain of 1%, and the variation in storage modulus was recorded for up to 200 s in a modular compact rheometer (Physica MCR 301, Anton Paar, Germany) with a parallel plate PP25 and a measuring distance of 0.5mm.

3.1.5 Water content of GelMA hydrogel

The 5% GelMA solution was prepared by dissolving 250 mg of GelMA in 4.5 mL of PBS and adding 0.5mL of 5% (w/v) Irgacure-2959. Using custom-made Teflon mold to cast samples of dimensions 10 x 2 mm, GelMA hydrogel discs (n=3) were prepared and the hydrogels were then subjected to lyophilization, and the dry weight (W_d) was noted using Sartorius electronic balance (model BSA 4202S-CW, Germany). Subsequently, the hydrogel was transferred to a 12-well plate and incubated in 1mL of PBS at 37 °C for 24 h under sterile conditions. The

samples were taken from the well and any excess liquid was blotted, and the wet weight (W_s) was measured to determine the water content of the hydrogel. The water content (%) in 5 % GelMA hydrogel was calculated using equation

$$\text{Water content \%} = \frac{(W_s - W_d)}{W_s} \times 100 \dots\dots\dots \text{Equation 2}$$

3.1.6 Stability analysis of GelMA hydrogel

The stability analysis was conducted under sterile conditions to avoid degradation of test samples by microbial contamination. The 5% GelMA hydrogel discs were prepared using custom made Teflon mold as previously described. The hydrogel samples ($n=3$) were lyophilized and the dry weight (W_i) was noted. The lyophilized samples were transferred to 1 mL PBS taken in 12-well plate and incubated at 37 °C for the durations of days 1, 3, and 7. The hydrogel samples on days 1, 3 and 7 were lyophilized and noted dry weight (W_t). The weight loss in percentage of 5 % GelMA hydrogels were calculated using the equation given below.

$$\text{Weight loss\%} = \frac{W_i - W_t}{W_i} \times 100 \dots\dots\dots \text{Equation 3}$$

3.1.7 Sterilization of GelMA

Lyophilized GelMA samples were cut into small pieces and packed in Medical grade EtO sterilizer rolls (250mg/pack) and sterilized by passing Ethylene Oxide gas (3M Steri-Vac™ Sterilizer/Aerator Model 8XL) at 37 °C. After the sterilization process, the GelMA samples were analyzed for the presence of EtO residues using Gas Chromatography (GC). To do this, the GelMA samples were

carefully weighed and placed in clean headspace vials containing 2 mL of deionized water. The vials were securely sealed and incubated at a temperature of 37 °C for a duration of 4 h to allow for extraction of any remaining EtO. Once the extraction was complete, the vials were subjected to GC analysis. The peak area of EtO at a room temperature of 3 min is integrated and quantified by comparing with the peak area of known concentrations of the certified reference material (2000 µg/mL of EtO in Dichloromethane).

The ability of GelMA to solubilize and retain its crosslinking property even after EtO sterilization was also examined using the tube invert method (Komeri *et al.*, 2017). A 15 mL glass vial containing 3 mL of 5% GelMA prepared in serum free media and containing 0.5% Irgacure2959 were exposed to UV light with a wavelength of 365 nm. The vial was then inverted every 60 s to observe the flow. The cessation of flow on vial inversion was used to confirm the crosslinkability of GelMA.

3.2 Rheological property of GelMA solution

The GelMA solution of 5% (w/v) was prepared by dissolving 500 mg of lyophilized polymer in 9 mL serum free media (Minimal Essential Media, Glutamine, Sodium bicarbonate, Pen-strep) at a temperature of 37 °C. The photoinitiator, Irgacure-2959 at a concentration of 5% (w/v) was prepared in PBS by heating in a water bath at 80 °C, and from that 1 mL was added to the GelMA solution. The solution was thoroughly mixed by pipetting and incubated at 37 °C until analysis. The rheological properties were analyzed using a rheometer

(Physica MCR 301, Anton Paar, Germany) using a parallel plate with geometry of 25 mm diameter and at a measuring distance of 0.5 mm.

Temperature sweep test of GelMA solution were performed. The sol-gel transition of 5% GelMA was studied by conducting a temperature sweep test in rotational mode. Approximately 0.4 mL of 5% (w/v) GelMA was placed between the parallel plates at 0.5 mm space. The temperature of the sample stage was gradually reduced from 20 °C to 8 °C at a rate of -1°C per minute. The viscosity of the solution was measured at 1 min interval when the temperature of the sample was reduced to -1°C. The specific temperature at which gelation occurred was determined by analyzing the log viscosity verses temperature curve using GraphPad Prism Version 9.0 software (GraphPad Software Inc, San Diego, CA, USA).

3.3 Formulation of GelMA-Gelatin (GelMA-G) polymer solution

The GelMA-gelatin polymer solution was prepared by dissolving 500 mg GelMA and 300 mg gelatin in 9 mL MEM serum free media at 37 °C by incubating in a water bath. A stock concentration of 5% (w/v) Irgacure-2959 was prepared in PBS by heating in a water bath at 80 °C and from that solution, 1 mL was added to the polymer solution. Thus, the bioink formulation comprising of 5% GelMA, 3% gelatin and 0.5% Irgacure 2959 denoted as GelMA-G were used for further analysis.

3.4 Rheological properties of GelMA-G formulation

3.4.1 Temperature sweep analysis

Temperature sweep tests were conducted to determine the physical gelation temperature of 5% GelMA with 3% gelatin formulation. The sol-gel transition of GelMA-G formulation was studied by conducting a temperature sweep test in rotational mode. About 0.4 mL of GelMA-G solution was placed between the parallel plates at 0.5 mm space and the temperature of the sample stage was gradually reduced from 24 °C to 8 °C at a rate of -1°C per minute. The variation in viscosity of the solution with respect the change in temperature was measured. The specific temperature at which gelation occurred was determined by analyzing the log viscosity verses temperature curve using GraphPad Prism Version 9.0 software (GraphPad Software Inc, San Diego, CA, USA).

3.4.2 Dynamic viscosity analysis

The dynamic viscosity analysis helps in determining the shear-thinning behavior of GelMA-G polymer solution. The test for dynamic viscosity analysis was performed in rotational mode. About 0.4 mL of sample were placed between the parallel plates and at constant temperature (20 °C) a dynamic shear rate from 1 to 1000/s was applied for the analysis. Thus, the variation in viscosity of 5% GelMA-3% gelatin solution with respect to the increase in shear rate was analyzed.

3.4.3 UV curing analysis

The UV curing analysis was performed at 20 °C in the oscillatory mode at a constant frequency (10 Hz) and strain (1%) to investigate the photo-

polymerization of GelMA with gelatin formulation. The samples were exposed to 365 nm UV light source Omnicure 1000 (Lumen Dynamix LDGI) and the variation in storage modulus with respect to UV time was recorded up to 200 s.

3.5 *Biological evaluation of GelMA-G formulation*

3.5.1 *In vitro* cytotoxicity evaluation

The cytotoxicity of the GelMA-G formulation was evaluated by direct contact and an extraction method based on ISO 10993-5, 2009.

3.5.1.1 *Culture of L-929 cells*

Mouse Fibroblast cells (L-929, ATCC) were maintained in Minimal Essential Medium (Merck-Sigma, Cat. # M0769, Germany) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Cat. #10270106, USA) and 1% penicillin-streptomycin antibiotics (10000 IU Penicillin and 10000 µg/mL Streptomycin, Gibco, Cat. #15070-063, USA) in a carbon dioxide (CO₂) incubator (New Brunswick, Galaxy 170 S, Canada) set at 37 °C, 5% CO₂, and >90% relative humidity.

The culture media was replaced with fresh medium on alternate days until sub confluency. The cells were then sub-cultured by trypsin treatment (Trypsin-EDTA (0.25%), phenol red, Gibco, Cat. #25200056) at a split ratio of 1:2. During subculturing, the culture media was removed from the culture flask and gently rinsed the cells with 1X PBS (0.01M, pH 7.4). After discarding PBS, appropriate volume of pre-warmed Trypsin – EDTA was added and incubated for 3-5 min in CO₂ incubator. The cells were then viewed under microscope to see whether the

cells are detached from the flask and complete media with double the volume of trypsin was added into the flask to inactivate trypsin action. The medium containing cells were collected into a 15mL centrifuge tube and centrifuged at 1500 rpm for 2 min ((Eppendorf 5430R, India). The supernatant was discarded and the pellet was resuspended in fresh media and transferred into culture flask for maintenance of cells and seeded onto multi-well plates for cytotoxicity test.

3.5.1.2 Preparation of GelMA-G hydrogel

The GelMA-G formulation with 5% GelMA, 3% gelatin and 0.5% Irgacure was prepared as previously described in section 3.3. The GelMA solution was transferred to a sterile petri dish of diameter 60 mm. The petridish was then exposed to UV light of 365 nm for 3 min to crosslink the GelMA-G solution and form hydrogel sheets of thickness 2 mm. For the cell culture experiments, GelMA-G hydrogels were prepared under aseptic conditions. The samples for direct contact test (disc of diameter = 4 mm) and test on extract (disc of diameter = 8 mm and height = 2 mm) were prepared by cutting cylindrical discs of required size from GelMA-G hydrogel sheets using sterile plastic punching tool.

3.5.1.3 Direct contact test

L-929 cell monolayer was trypsinized and the cell suspension were prepared. The cells were then counted using Neubauer hemocytometer and the cell suspension was diluted to get cell number of 1×10^6 cells/mL. The cells were seeded onto 24 well plate at a concentration of 30,000 cells/well and incubated in CO₂ incubator for 24 h. Negative and positive cytotoxic controls used were ultra-

high-molecular-weight polyethylene (UHMWPE) and organo-tin stabilized polyvinyl chloride (PVC) respectively. Triplicate samples of GelMA-G and controls were placed on an L-929 monolayer and incubated at 37 °C for a period of 24 h. The cells surrounding the samples were observed under an inverted phase contrast microscope (Nikon Eclipse TS2-FL, Japan), and the cytotoxicity was assessed based on changes in general cell morphology, vacuolization, detachment, cell lysis and membrane integrity.

Table 2: Cytotoxicity reactivity grading for direct contact test

Grade	Reactivity	Description
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending specimen size up to 0.33 cm
4	Severe	Zone extending farther than 0.33 cm beyond specimen

3.5.1.4 Test on extract and MTT Assay

Cytotoxicity was evaluated by exposing cells to the extract of GelMA-G hydrogel. The hydrogel discs were incubated in a shaking incubator (Orbitek, India) at 37 °C for 24 h with culture medium containing serum, at a ratio of 1.25 cm²/mL. Various dilutions of GelMA-G neat extract (50%, 25%, and 12.5%) were obtained by diluting the 100% extract with MEM medium containing FBS. Phenol prepared by diluting phenol stock solution (13 mg/mL) to 1.3 mg/mL with culture medium containing serum was used as positive cytotoxic control.

L-929 cells were seeded in a 96-well plate at a density of 1×10^5 cells per well and allowed to reach sub-confluency. Subsequently, 100 µL of each GelMA-

G extract dilution (n=8) was added to L-929 cells. Positive cytotoxic controls were treated with 100 µL of diluted phenol and cells incubated in normal culture medium were considered as cell control. After 24 h of incubation, the response of the cells to the extract treatment was analyzed under an inverted phase contrast microscope (Nikon Eclipse TS2-FL, Japan). The viability of cells exposed to extract was determined using the MTT assay. After incubating cells for 24 h, the extract and control media were replaced with 50 µL of MTT solution (1 mg/mL in serum-free MEM) (Merck-Sigma, Germany). The cells were then incubated at 37 °C in a CO₂ incubator for 2h. The MTT solution was then replaced with 150 µL isopropanol and the plate was thoroughly shaken to dissolve the formazan crystal. The absorbance was measured at 570 nm using a multi-well plate reader (Biotek, Powerwave XS, USA). The OD of the samples was taken with Gen5 software and exported to Microsoft Excel. The values of GelMA-G extract and positive control were normalized with the cell control and represented as percentage metabolic activity as per the following equation.

$$\% \text{ Metabolic activity of cells} = \frac{\text{OD of test samples}}{\text{Average OD of cell control}} \times 100 \dots\dots\dots \text{Equation 4}$$

Where OD represent the optical density

3.5.2 Cytocompatibility evaluation of GelMA-G formulation

An ideal bioink should possess a combination of the mechanical, including rheological, and crosslinking properties for ensuring shape fidelity and structural maintenance of the bioprinted product, while cytocompatibility ensures the viability and maintenance of the cells within the bioink. Therefore, it is also

essential to evaluate the cytocompatibility of the GelMA-G formulation to ensure it meets the requirements for use as a bioink.

3.5.2.1 *HepG2 cell culture*

Human hepatocellular carcinoma cell line, (HepG2, National Centre for Cell Science, India) were cultured in Minimum Essential Medium (MEM Merck-Sigma, Germany) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, USA) and 0.1% Penicillin-Streptomycin antibiotic (Pen-Strep, USA). The culture conditions were maintained at 37 °C, 5% CO₂, and >90% relative humidity. Once the cells attain confluency, the cells were trypsinized by incubating with Trypsin-EDTA for 5 min. After incubation, fresh media was added into the flask and the cell suspension was centrifuged at 1500 rpm for 2 min. The pellet was resuspended in culture media.

3.5.2.2 *Cell adhesion*

The adhesion and morphology of HepG2 cells on the GelMA-G hydrogel was studied by actin staining. For seeding the HepG2 cells on GelMA-G hydrogel, sterile cover glasses were transferred aseptically into a 12 well plate and around 100 µL of GelMA-G solution was added into the cover glass and spread uniformly. The GelMA-G solution was then removed and the cover glass was exposed under UV for 3 min for coating the cover glass with GelMA-G. From the cell suspension, HepG2 cells at a density of 3×10^5 cells/well were seeded on 12 well plate containing cover glass coated with GelMA-G hydrogel. After 72 h of culture, the culture medium was removed, and the cells attached on the coated cover glass

were washed three times with PBS. The cells were fixed in 4% paraformaldehyde for 30 min. The fixative was removed and cells were washed with PBS. The cells were then permeabilized by treating with 0.1% Triton-X 100 in PBS for 3 min. Rhodamine-phalloidin (diluted 1:100 in PBS) was added on cells and incubated for 15 min at room temperature. The nucleus of the cells was counterstained with Hoechst 33258 (0.05 $\mu\text{g/mL}$ in PBS) for 1 min. The cells were observed under a fluorescence microscope (Leica DMI 6000, Switzerland) using filters N21 (red emission) and A (blue emission). Images for actin and nucleus was separately captured using Leica Application Suite (LAS V3.8) through DFC 350 monochrome camera and pseudo-colored.

3.5.2.3 *Cell encapsulation and viability*

A sub-confluent culture of HepG2 cells were trypsinised and the cell suspension was centrifuged at 1500 rpm for 2 min and cell pellet at a concentration of 9×10^5 cells was resuspended in 750 μL of GelMA-G solution by gentle pipetting. HepG2 cells at a density of 3×10^5 cells in 250 μL of GelMA-G was transferred into a 24 well plate and was crosslinked under UV for 3 min. The viability of cells encapsulated in GelMA-G hydrogel was analyzed by live/dead staining immediately after photo-crosslinking (0 h) and after culturing in MEM+10% FBS for 24 h cell encapsulated hydrogels were incubated in 0.01% Fluorescein diacetate prepared in serum free medium for a duration of 4 min (FDA Merck- Sigma, Germany) and Propidium Iodide (PI, HiMedia, Cat#TC252, India) at a concentration of 0.005% was added into the FDA containing medium in cells and incubated for 1 min. After incubation, hydrogels were washed with serum free

medium and the cells were then observed under a fluorescence microscope using I3 (green emission for live cells) and N21 (red emission for dead cells) filters.

3.5.2.4 *Cell encapsulation and proliferation*

HepG2 cells were encapsulated in GelMA-G hydrogel as described before with a cell concentration of 3×10^5 cells. Cell proliferation was analyzed using Cell Counting Kit – 8 (CCK-8, Sigma) on days 1 and 7 with a gap of at least one cell division. On specified days, the culture medium was replaced with the CCK-8 medium (1:10 dilution in serum-free medium) and incubated for 2 h. The CCK-8 medium was transferred into a fresh 96 well plate and the absorbance were measured at 450 nm using a multi-well plate reader (Biotek, USA). The proliferation was calculated as percentage of cell activity normalized to the absorbance value of day 7 with the average absorbance value obtained on day 1.

3.6 *Printability assessment of GelMA-G formulation*

The printability of 5% GelMA, 3% gelatin formulation for extrusion-based 3D bioprinting was assessed by evaluating its extrudability, ability to dispense different single layer patterns and the ability to print multilayer constructs. All printability assessments were performed after the room temperature was adjusted to 18-20 °C.

3.6.1 *Extrudability*

The extrudability of GelMA-G bioink formulation was primarily evaluated using a filament extrusion test which involves extruding a filament into the air rather than on a substrate. A freshly prepared 5% GelMA-3% gelatin solution was

filled into a 5mL cartridge and subjected to thermal gelation by keeping at 4 °C for about 5 min. The cartridge was then connected to a tapered-shaped plastic printing nozzle with an inner orifice diameter of 410 µm. The cartridge was then loaded onto the pneumatic extrusion print head of 3D bioprinter (3D Discovery, RegenHU, Switzerland). The pressure for extrusion was gradually increased at an increment of 0.05 MPa until a continuous flow of filament was observed.

3.6.2 Single and multilayer layer pattern printing

The printability of the GelMA-G formulation was evaluated by printing single layer of diverse patterns such as squares, triangles, and zigzags. The dimensions of patterns were investigated by measuring the width of the extruded filament. The designs for these patterns were created using BioCAD software (RegenHU, Switzerland). The single layer patterns were then printed on polystyrene dish using the pneumatic extrusion head of the 3D bioprinter. During printing, first the filament was extruded by applying the pressure required for continuous filament dispersion. A pressure of 55 kPa was found to be optimal and was applied to print patterns. Digital images of these patterns were taken for assessment.

A square grid multilayer pattern with outer dimensions of 10×10×2 mm and having internal perpendicular filaments 90 ° rotated on alternate layers with interfilament distance of 1 mm was created using 3D BioCAD software. Cartridge containing the GelMA-G solution was kept at 2-8 °C for 2-3 min to allow for physical gelation and the cartridge was loaded into a 3D printer and were printed based on the selected design. The constructs were printed at a speed of 10 mm/s

using pneumatic extrusion print head of 3D printer (3D Discovery, RegenHU, Switzerland) through a nozzle of internal diameter 410 μm . The 3D printed multilayer constructs were exposed to 365 nm UV light for 3 min for crosslinking. The crosslinked GelMA-G hydrogel was transferred aseptically to MEM complete medium and incubated at 37 °C in a CO₂ incubator for a period of 7 days. Digital images of the constructs at different time points (day 0, 1, 3 and 7) were then captured to assess the shape of the constructs.

The water content in the 3D printed construct over incubation in culture media at 37 °C was also assessed to evaluate the shrinking or swelling properties of the construct. The multilayer GelMA-G constructs (n=4) were printed and crosslinked under UV for 3 min and incubated in MEM complete medium at 37 °C in a CO₂ incubator. The wet weight (W_i) of the constructs after 24 h of incubation (day 1) was noted and the constructs were freeze-dried and its dry weight (W_d) was noted. Similarly, wet and dry weight of the constructs at days 3 and 7 was noted. The water content (%) in GelMA-G constructs at different time points (day 1, 3 and 7) was calculated using equation.

$$\text{Water content \%} = \frac{(W_i - W_d)}{W_i} \times 100 \dots\dots\dots \text{Equation 5}$$

3.6.3 Stability of 3D printed multilayer construct

The stability of the 3D printed GelMA-G multilayer construct with respect to the variation in crosslinking time was studied by analyzing the weight loss of constructs over incubation in MEM culture media at 37 °C. Square grid constructs of 10×10×2 mm was 3D printed as previously described and the constructs (n=4)

were crosslinked under UV for a duration of 3 min and 5 min. The initial weight (W_i) of the constructs were noted and the constructs were incubated in MEM complete medium at 37 °C in a CO₂ incubator for 7 days. The final weight (W_f) of constructs was measured after incubation on days (1, 3, and 7). The percentage of weight loss of the construct was calculated using the following equation:

$$\text{Weight loss (\%)} = \frac{W_i - W_f}{W_i} \times 100 \dots\dots\dots \text{Equation 6}$$

3.7 Hemocompatibility evaluation of 3D Printed construct

In vitro hemocompatibility of GelMA-G hydrogel was evaluated by haemolysis test according to ISO standard 10993-4,2017. A 3D design of cylindrical shape with diameter of 1.5 cm and 0.4 cm height and interfilament space of 1.5 mm was designed using BioCAD software (RegenHU, Switzerland). The cylindrical constructs were 3D printed using GelMA-G formulation and the printed samples were crosslinked under UV light for 5 min. The samples (n=3) were immersed in PBS contained in a fresh polystyrene 24-well plate and was agitated for 5 min. The PBS was aspirated out and 2.5 mL of human blood from a healthy volunteer was added to each hydrogel sample. About 0.5 mL of blood was taken immediately for measuring total hemoglobin using automatic hematology analyzer (ERBA Mannheim-H360, USA). The hydrogel samples were incubated in the remaining blood for 30 min under agitation at 70 ± 5 rpm inside a shaker incubator (Environ, Lab-Line, India) set at 35 ± 2 °C. Blood added to wells without any samples were considered as negative control. After incubation, the blood samples were centrifuged at $2500 \times g$ for 15 min and platelet poor plasma was

aspirated. The free hemoglobin liberated in to the plasma after exposure to hydrogel samples was measured using diode array spectrophotometer (Hewlett-Packard 8053, USA). The percentage hemolysis was calculated as per ASTM F756-17 standard.

$$\% \text{ Hemolysis} = \frac{\text{Free plasma hemoglobin conc.} \left(\frac{\text{mg}}{\text{mL}} \right)}{\text{Total hemoglobin conc.} \left(\frac{\text{mg}}{\text{mL}} \right)} \times 100 \dots\dots\dots \text{Equation 7}$$

3.8 3D Bioprinting of liver constructs with GelMA-G bioink formulation

3.8.1 Base bioink formulation

The GelMA-G formulation was prepared by dissolving 500 mg GelMA and 300 mg gelatin in 9 mL MEM serum free medium at 37 °C. A stock concentration of 5% (w/v) Irgacure-2959 was prepared in PBS by heating in a water bath at 80 °C and from that solution, 1 mL was added to the polymer solution. The final bioink formulation for 3D bioprinting liver was constituted by dispersing HepG2 cells at a concentration of 2.5×10^6 cells/mL of GelMA-G.

3.8.2 Extrusion 3D bioprinting

A square grid pattern with perpendicular internal filaments having 10x10x2 mm dimensions was designed in 3D BioCAD software (regenHU, Switzerland). G-code for the design was generated. The bioink was loaded into 5 mL cartridge and kept at 2-8 °C for 2-3 min for physical gelation. The cartridge was connected to the micro-extrusion head of 3D bioprinter (regenHU, 3D Discovery, Switzerland). Multiple constructs with HepG2 cells (6×10^5

cells/construct) were printed in a 12 well plate under a pneumatic pressure of 55 kPa and at a velocity of 10 mm/s. The constructs were crosslinked by irradiating UV light for 5 min. The distribution of the cells within the construct immediately after bioprinting were observed under phase contrast inverted microscope. The constructs were cultured in MEM medium containing FBS at 37 °C in CO₂ incubator for over a period of 7 days. The spent culture medium at regular intervals such as day 1, 3 and 7 were collected for analyzing liver-specific functions. The viability of cells within the bioprinted construct on 7 days of culturing was analysed by FDA/PI staining.

3.8.3 Liver specific functional evaluation

3.8.3.1 Albumin assay

Albumin synthesis by HepG2 cells was analysed using an ELISA kit (Bethyl Laboratories, Cat. No. E80-129, USA) according to the manufacturer's instructions. Albumin standards were prepared as shown in Table 3.

Table 3: Preparation of Albumin standards using Human reference serum

Standards	ng/mL	Volume of Albumin	Sample dilute
Initial	10,000	5 μ L	11mL
Std 1	400	100 μ L from initial	2.4mL
Std 2	200	500 μ L from Std 1	500 μ L
Std 3	100	500 μ L from Std 2	500 μ L
Std 4	50	500 μ L from Std 3	500 μ L
Std 5	25	500 μ L from Std 4	500 μ L
Std 6	12.5	500 μ L from Std 5	500 μ L
Std 7	6.25	500 μ L from Std 6	500 μ L
Std 8	0	-	500 μ L

Serial dilutions of standards and the spent culture medium collected at various time points were added in duplicate to the primary antibody (100 μ L)

coated 96 wells. After 1 h incubation at room temperature, wells were rinsed four times with ELISA wash buffer (50mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) and the secondary antibody (100 μ L) was added. After 1 h incubation, the wells were rinsed again and sequentially treated with 100 μ L of horse radish peroxidase solution and incubated for 1 h. The wells were then rinsed with wash buffer and 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added into each well and incubated for 30 min in the dark. The substrate reaction develops a blue color and the reaction was stopped by adding 100 μ L H₂SO₄ (0.18 M). Absorbance was measured at 450 nm in a multi-well plate spectrophotometer (Biotek, Powerwave XS, USA). The human albumin standard curve was plotted from the average absorbance values of each standard concentration and the albumin concentration in the spent culture medium collected at specific time points are calculated from the prepared standard curve. The concentration of albumin synthesized by the cells was calculated from the standard curve and presented as the mean $\pm$ standard deviation.

3.8.3.2 Urea assay

The ability of 3D bioprinted constructs with HepG2 cells was analyzed by estimating urea production in the spent medium using commercially available urea assay kit (Biochain, USA). The kit utilizes a chromogenic reagent that forms a colored complex specifically with urea and the intensity of the color formed is directly proportional to the concentration of urea in the sample.

50 μ L of culture medium collected from the bioprinted construct at various time points, urea standard (5mg/dL), and control culture medium (blank) was

added in triplicate to a 96 well plate. 200 μ L of working reagent provided in the kit was then added to each well and incubated for 50 min. The absorbance was measured at 450 nm in a multi-well plate spectrophotometer (Biotek, Powerwave XS, USA) and the concentration of urea in the culture media was calculated using the equation and presented as the mean $\pm$ standard deviation.

$$\text{Concentration of urea} = \frac{\text{OD of sample} - \text{OD of blank}}{\text{OD of standard} - \text{OD of blank}} \times \frac{5\text{mg}}{\text{dL}} \dots\dots\dots \text{Equation 8}$$

Where OD is the optical density

3.9 Phototoxicity analysis in GelMA encapsulated cells

The impact of UV on cells encapsulated in GelMA was studied by measuring the intracellular ROS (iROS) levels using the DCFDA-cellular ROS assay (Abcam ab113851).

A sub-confluent culture of HepG2 cells were trypsinized and the cell pellet was incubated with 25 μ M DCFDA prepared in serum free medium for 45 min at 37 $^{\circ}$ C in dark. The cells were then washed with PBS by centrifuging at 1500 rpm for 2 min. The GelMA bioink was prepared by mixing a total of pre-stained 9×10^5 HepG2 cells with 600 μ L of GelMA solution. This bioink solution was transferred into a 24 well plate at a volume of 200 μ L/well and exposed to UV irradiation of 365 nm for 3 min. The GelMA got crosslinked and HepG2 cells were encapsulated within the hydrogel. The cells cultured in normal TCPS multiwell plate was considered as assay control. The pre-stained HepG2 cells were transferred into a 24 well plate (3×10^5 cells/well) and exposed to UV for 3 min. The cells exposed to UV was considered as positive control. Similarly, HepG2 cells were also seeded

on 24 well plate that were not exposed to UV and was considered as negative control. The constructs were transferred into 1.5 mL vial and HepG2 encapsulated in the GelMA hydrogel was harvested by incubating with 200 μ L of 0.3% collagenase – A prepared in calcium containing HEPES buffer (100 mM HEPES buffer with 20 mM calcium chloride) for 20 min at 37 °C in water bath. After complete dissolution of hydrogel, the cell suspension was transferred into 1.5 mL vial and centrifuged at 1500 rpm for 2 min (Eppendorf 5430R, India). The control cells also were treated with collagenase as done for the encapsulated cells and centrifuged. The cell pellets obtained from test and assay controls were resuspended in 500 μ L PBS and centrifuged at 1500 rpm for 2 min. The PBS was then discarded and the cells were again resuspended in 500 μ L of fresh PBS. The cell suspension was transferred into a black-clear 96 well plate (Greiner Bio-One). The fluorescence intensity of 2', 7' – dichlorofluorescein (DCF), which is produced from DCFDA as a result of oxidization by iROS activity, was measured using a multiwell plate reader (Synergy H1, Biotek, USA) at excitation/emission wavelengths of 485 nm/535 nm. The percentage of ROS activity was determined from the relative fluorescence unit (RFU) of cells retrieved from GelMA and the positive control samples by normalizing them against the negative control.

$$\% \text{ ROS activity} = \frac{\text{Test RFU}}{\text{Negative control RFU}} \times 100 \dots\dots\dots \text{Equation 9}$$

3.10 Formulation of bioink loaded with Antioxidant (AO) cocktails

Two antioxidant vitamin, α -Tocopherol (α -Toc) and Ascorbic acid (AA) were chosen to formulate GelMA-G with Antioxidant cocktail system.

3.10.1 Dose response of α -Tocopherol on HepG2 cells

HepG2 cells were seeded into a 96 well plate at a concentration of 1×10^5 cells/well and allowed to attain sub confluency inside a CO₂ incubator. A stock solution of α -Tocopherol was prepared at a concentration of 10 M in pure ethanol. A second stock was prepared by diluting in PBS to obtain a concentration of 5 M. The solution was added directly to MEM culture media to get a final concentration of 1 M. A logarithmic range of α -Toc (1000, 100, 10, 1 and 0.1 mM) were prepared in MEM culture media. The culture medium from sub confluent HepG2 cells was replaced with various doses (1000, 100, 10, 1 and 0.1 mM) of α -Tocopherol. The cells in normal culture medium were considered as cell control. The plate was incubated at 37°C for 24 h in a CO₂ incubator. The medium from cells were removed and 50 μ L of MTT in serum free medium (1 mg/mL) was added and incubated for 2 h at 37 °C. The MTT medium was removed and the formazan product formed was dissolved in isopropanol and absorbance was measured using a microplate reader (Biotek, USA) at 570 nm. The half maximal inhibitory concentration (IC₅₀) was calculated from the optical density (OD) using GraphPad Prism Version 9.0 software with built in module (GraphPad Software Inc, San Diego, CA, USA). The OD values were transformed into Log (x) values and was normalized with the control. Then the IC₅₀ was obtained using the option Non-linear regression, curve fit.

3.10.2 Crosslinking of GelMA-G in presence of antioxidants

The GelMA-G formulation was prepared by dissolving 250 mg GelMA and 150 mg gelatin in 4.5 mL serum free medium at 37 °C. 1 mL of 5% Irgacure

2959 was then added into GelMA-G solution and mixed thoroughly. The antioxidants selected for the formulation were added into the GelMA-G solution.

The concentration of Ascorbic acid (AA), one of the components in the antioxidant cocktail formulation selected was 3.4 mM, based on a previous report. The ascorbic acid stock solution was prepared by dissolving in serum-free media at a concentration of 56.7 mM. The ascorbic acid was then added to the GelMA-G solution in such a way to achieve a final concentration of 3.4 mM Ascorbic acid (i.e. 60 μ L of AA in 940 μ L of GelMA-G). To prepare the second formulation, GelMA with α -Tocopherol, a stock concentration of 1 M α -Toc was prepared in ethanol, and then a 100mM α -toc solution (second stock) was prepared in PBS. α -toc was added to GelMA-G from the 100 mM solution to attain a final concentration of 100 μ M (i.e. 1 μ L of α -Toc in 999 μ L of GelMA-G). GelMA-G with AO cocktail system was prepared by adding ascorbic acid and α -tocopherol into GelMA-G in such a way to get 3.4mM AA and 100 μ M α -toc (i.e. 60 μ L of AA + 1 μ L of α -Toc in 939 μ L of GelMA-G). 200 μ L of each solution was transferred to a custom-made Teflon mold of cylindrical shape with diameter 10 mm and height 2 mm. The hydrogel precursor solution was then exposed to UV light for 5 min and the crosslinked hydrogels were taken out and digital images of the hydrogels were captured.

3.10.3 Analysis of photoprotective property of GelMA-AO

To evaluate the photoprotective effect of GelMA with AO cocktail system, iROS levels after photo-crosslinking were measured using the DCFDA-cellular ROS assay (Abcam ab113851), as described before. The experiment contained

four hydrogel systems: GelMA-G, GelMA-G + AA, GelMA-G + α -Toc, and GelMA-G + AA + α -Toc. The base GelMA-G formulation was prepared as described in the previous section and each bioink formulations were prepared as shown in the Table 4.

Table 4: Preparation of bioink formulations

Bioink formulations	GelMA-G solution	Ascorbic acid (56.7mM)	α-Tocopherol (100 mM)
GelMA-G	600 μ L	-	-
GelMA-G+AA	564 μ L	36 μ L	-
GelMA-G+ α -Toc	599.4 μ L	-	0.6 μ L
GelMA-G+AA+ α -Toc	563.4 μ L	36 μ L	0.6 μ L

The four bioink systems were prepared by adding a total of DCFDA pre-stained 9×10^5 HepG2 cells/ 600 μ L of each formulation. All the bioink formulations were then transferred into a 24 well plate (200 μ L/well). The formulations were exposed to UV for 5 min in order to encapsulate the cells within the hydrogels. The HepG2 encapsulated in the four GelMA-G formulations were harvested by incubating with 200 μ L of 0.3% collagenase – A prepared in calcium containing HEPES buffer (100 mM HEPES buffer with 20 mM calcium chloride) for 20 min at 37 °C. After complete dissolution of hydrogels, the cell suspension was centrifuged at 1500 rpm for 2 min (Eppendorf 5430R, India). The cell pellets obtained were resuspended in 500 μ L PBS and centrifuged at 1500 rpm for 2 min. The PBS was then discarded and the cells were again resuspended in 500 μ L of fresh PBS. The cell suspension was transferred into a black-clear 96 well plate (Greiner Bio-One). The UV protective property of GelMA-G with antioxidant formulations was analyzed by measuring the fluorescence intensity of 2', 7' –

dichlorofluorescein (DCF), which is produced from DCFDA as a result of oxidization by iROS activity, using a multi-well plate reader (Synergy H1, Biotek, USA) at excitation/emission wavelengths of 485 nm/535 nm. The percentage of ROS activity within each GelMA-G antioxidant formulation (test groups) was determined from the relative fluorescence unit (RFU) of cells retrieved from test groups by normalizing them against the base GelMA-G formulation (control group).

$$\% \text{ ROS activity} = \frac{\text{Test RFU}}{\text{GelMA RFU}} \times 100 \dots\dots\dots \text{Equation 10}$$

3.10.4 Cell viability assay

The viability of the cells encapsulated in GelMA-G and GelMA-G-AA+ α -Toc (denoted as GelMA-G-AO) was determined by Alamar blue assay. A stock concentration of 0.5% Alamar blue reagent (Resazurin Sodium Salt) was prepared by dissolving in PBS and a working concentration of 0.005% was then prepared in serum-free medium.

A sub-confluent culture of HepG2 cells were trypsinised and the cell suspension was centrifuged at 1500 rpm for 2 min. The cell pellet at a concentration of 9×10^5 was then resuspended in 600 μ L of GelMA-G and GelMA-G-AO solution by gentle pipetting. Then the bioink solutions were transferred into a 24 well plate (200 μ L/well) as triplicates and subjected to UV light irradiation for 5 min to encapsulate the cells within the hydrogel and cultured in MEM medium containing FBS for 24 h in CO₂ incubator at 37 °C. After 24 h of incubation, culture media from each well was replaced with 500 μ L/well of

0.005% Alamar blue solution and incubated for 4h in CO₂ incubator at 37 °C. The Alamar blue medium was then transferred to a fresh 96 well plate (150 µL/well) and the absorbance was measured at 570 nm and at 600 nm as the reference wavelength using a multi-well plate reader (Biotek, USA). From the absorbance value obtained cell viability % was calculated using equation

$$\text{Cell viability \%} = \frac{(O2 \times A1) - (O1 \times A2)}{(O2 \times P1) - (O1 \times P2)} \times 100 \dots\dots\dots \text{Equation 11}$$

where O1-Molar extinction coefficient at 570 nm

O2- Molar extinction coefficient at 600 nm

A1-Absorbance of GelMA-G-AO sample at 570 nm

A2- Absorbance of GelMA-G-AO sample at 600 nm

P1- Absorbance of GelMA-G sample at 570 nm

P2- Absorbance of GelMA-G sample at 600 nm

3.11 Biological evaluation of GelMA-G-AO formulation

3.11.1 Preparation of GelMA-G-AO hydrogel

The GelMA-G-AO hydrogels were prepared by dissolving 250 mg GelMA and 150 mg gelatin in 4.195 mL serum-free medium (MEM) at 37 °C. Then 300 µL of Ascorbic acid and 5 µL of α-Tocopherol was added in such a way to obtain a final concentration of 3.4 mM AA and 100 µM α-Toc respectively. 0.5 mL of 5% (w/v) Irgacure 2959 was also added into the GelMA-G-AO solution and mixed thoroughly.

3.11.2 *In vitro* cytotoxicity evaluation

The cytotoxicity of the GelMA-G-AO hydrogel was evaluated by direct contact and test on extraction method based on ISO 10993-5,2009.

The GelMA-G-AO solution was then transferred to a sterile petri dish of diameter 60mm and exposed to UV light of 365 nm for 5 min for generating GelMA-G-AO hydrogel sheets. The samples for direct contact test (disc of diameter = 4 mm) and test on extract (disc of diameter = 8 mm and height = 2 mm) were prepared by cutting cylindrical discs of required size from GelMA-G-AO hydrogel sheets using sterile plastic punching tool.

3.11.2.1 *Direct contact test*

L-929 cell monolayer was trypsinised and the cell suspension were prepared. The cells were then counted using Neubauer hemocytometer and the cell suspension was diluted to get cell number of 1×10^6 cells/mL. The cells were seeded onto 24 well plate at a concentration of 30,000 cells/well and incubated in CO₂ incubator for 24h. Triplicate samples of GelMA-G-AO and positive (PVC) and negative (UHMWPE) controls were placed on an L-929 monolayer and incubated at 37 °C for a period of 24 h. The cells surrounding the samples were observed under an inverted phase contrast microscope (Nikon Eclipse TS2-FL, Japan), and the cytotoxicity was assessed based on changes in general cell morphology, vacuolization, detachment, cell lysis and membrane integrity.

3.11.2.2 Test on extract and MTT Assay

Cytotoxicity was evaluated by exposing cells to the extract of GelMA-G-AO hydrogel. The hydrogel discs were incubated in a shaking incubator (Orbitek, India) at 37 °C for 24 h with culture medium containing serum, at a ratio of 1.25 cm²/mL. Various dilutions of GelMA-G-AO neat extract (50%, 25%, and 12.5%) were obtained by diluting the 100% extract with MEM medium containing FBS.

L-929 cells were seeded in a 96-well plate at a density of 1×10^5 cells per well and allowed to reach sub-confluency. Subsequently, 100 μ L of each GelMA-G-AO extract dilution (n=8) were added to L-929 cells. Positive cytotoxic controls were treated with 100 μ L of diluted phenol and cells incubated in normal culture medium were considered as cell control. After 24 h of incubation, the response of the cells to the extract treatment was analyzed under an inverted phase contrast microscope (Nikon Eclipse TS2-FL, Japan). The viability of cells exposed to extract was determined using the MTT assay. The values of GelMA-G-AO extract and positive control were normalized with the cell control and represented as percentage metabolic activity as per the following equation.

$$\% \text{ Metabolic activity of cells} = \frac{\text{Absorbance of test samples}}{\text{Avg absorbance of cell control}} \times 100 \text{ ..Equation 12}$$

3.11.3 Cytocompatibility evaluation

The cytocompatibility of the GelMA-G-AO hydrogel was evaluated on HepG2 cells by cell adhesion, cell viability and cell proliferation.

3.11.3.1 Cell adhesion

The adhesion and morphology of HepG2 cells on the GelMA-G-AO hydrogel were studied by actin staining. For seeding the HepG2 cells on GelMA-G-AO hydrogel, sterile cover glasses were transferred aseptically into a 12 well plate and around 100 μ L of GelMA-G-AO solution was added into the cover glass and spread uniformly. The GelMA-G-AO solution was then removed and the cover glass was exposed under UV for 5 min for coating the cover glass with GelMA-G-AO. From the cell suspension, HepG2 cells at a density of 3×10^5 cells/well were seeded on 12 well plate containing cover glass coated with GelMA-G-AO hydrogel. After 72 h of culture, the culture medium was removed, and the cells attached on the coated cover glass were washed three times with PBS. The cells were fixed in 4% paraformaldehyde for 30 min. The fixative was removed and cells were washed with PBS. The cells were then permeabilized by treating with 0.1% Triton-X 100 in PBS for 3 min. Rhodamine-phalloidin (diluted 1:100 in PBS) was added on cells and incubated for 15 min at room temperature. The nucleus of the cells was counterstained with Hoechst 33258 (0.05 μ g/mL in PBS) for 1 min. The cells were observed under a fluorescence microscope (Leica DMI 6000, Switzerland) using filters N21 (red emission) and A (blue emission).

3.11.3.2 Cell encapsulation and viability

A sub-confluent culture of HepG2 cells were trypsinised and the cell suspension was centrifuged at 1500 rpm for 2 min and cell pellet at a concentration of 9×10^5 cells was resuspended in 750 μ L of GelMA-G-AO solution by gentle pipetting. HepG2 cells at a density of 3×10^5 cells in 250 μ L of GelMA-G-AO

was transferred into a 24 well plate and was crosslinked under UV for 3 min. The viability of cells encapsulated in GelMA-G-AO hydrogel was analyzed by live/dead staining immediately after photo-crosslinking (0 h) and after culturing in MEM+10% FBS for 24 h. For live/dead staining, cell encapsulated hydrogels were incubated in 0.01% Fluorescein diacetate prepared in serum free medium for a duration of 4 min (FDA Merck- Sigma, Germany) and Propidium Iodide (PI, HiMedia, Cat#TC252, India) at a concentration of 0.005% was added into the FDA containing medium in cells and incubated for 1 min. After incubation, hydrogels were washed with serum free medium and the cells were then observed under a fluorescence microscope using I3 (green emission for live cells) and N21 (red emission for dead cells) filters.

3.11.3.3 Cell encapsulation and proliferation

HepG2 cells were encapsulated in GelMA-G-AO hydrogel as described before with a cell concentration of 3×10^5 cells. Cell proliferation was analyzed using Cell Counting Kit – 8 (CCK-8, Sigma) on days 1 and 7 with a gap of at least one cell division. On specified days, the culture medium was replaced with the CCK-8 medium (1:10 dilution in serum-free medium) and incubated for 2 h. The CCK-8 medium was transferred into a fresh 96 well plate and the absorbance were measured at 450 nm using a multi-well plate reader (Biotek, USA). The proliferation was calculated as percentage of cell activity normalized to the absorbance value of day 7 with the average absorbance value obtained on day 1.

3.12 Rheological properties of GelMA-G-AO formulation

The GelMA-G-AO solutions were prepared by dissolving 500 mg GelMA and 300 mg gelatin in 8.39 mL serum-free medium (MEM) at 37 °C in a water bath. 600 µL of Ascorbic acid and 10 µL of α -Tocopherol was added into the GelMA solution to obtain a final concentration of 3.4 mM AA and 100 µM α -Toc respectively. 1 mL of 5% Irgacure 2959 was then added into the GelMA-G-AO solution and mixed thoroughly. Thus, the bioink formulation comprising of 5% GelMA, 3% gelatin, 3.4 mM Ascorbic acid, 100µM α -Tocopherol and 0.5% Irgacure 2959 were used for further analysis.

3.12.1 Temperature sweep analysis

Temperature sweep tests were conducted to determine the physical gelation temperature of 5% GelMA with 3% gelatin formulation. The sol-gel transition of GelMA-G formulation was studied by conducting a temperature sweep test in rotational mode. About 0.4 mL of GelMA-G solution was placed between the parallel plates at 0.5 mm space and the temperature of the sample stage was gradually reduced from 24 °C to 8 °C at a rate of -1°C per minute. The variation in viscosity of the solution with respect the change in temperature was measured. The specific temperature at which gelation occurred was determined by analyzing the log viscosity verses temperature curve using GraphPad Prism Version 9.0 software (GraphPad Software Inc, San Diego, CA, USA).

3.12.2 Dynamic viscosity analysis

The dynamic viscosity analysis helps in determining the shear-thinning behavior of GelMA-G polymer solution. The test for dynamic viscosity analysis was performed in rotational mode. About 0.4 mL of sample were placed between the parallel plates and at constant temperature (20 °C) a dynamic shear rate from 1 to 1000/s was applied for the analysis. Thus, the variation in viscosity of GelMA-G-AO solution with respect to the increase in shear rate was analyzed.

3.12.3 UV curing analysis

The UV curing analysis was performed at 20 °C in the oscillatory mode at a constant frequency (10 Hz) and strain (1%) to investigate the photopolymerization of GelMA-G with antioxidant formulation. The samples were exposed to 365 nm UV light source Omnicure 1000 (Lumen Dynamix LDGI) and the variation in storage modulus with respect to UV time was recorded up to 200s.

3.13 Printability assessment of GelMA-G-AO formulation

The printability of GelMA-G-AO formulation was primarily assessed by filament extrusion test followed by printing different patterns like square, triangle and zigzag to study the width size of the extruded filament, the ability of the bioink to print and maintain different angles. Similarly, a circular pattern was also printed to analyze the ability of bioink formulation to print curves. Square grid multilayer constructs with different dimensions such as 10×10×2 mm, 11×11×2 mm and 12×12×2 mm with internal perpendicular filament was printed to evaluate the ability of bioink to maintain the shape fidelity. All the patterns were created using

3D BioCAD (regenHU, Switzerland) software and G-codes were generated to print at a speed of 10 mm/s at 20 °C. Printing experiments were performed using extrusion print head of 3D printer (3D Discovery, RegenHU, Switzerland) with nozzle of size 410 µm. The GelMA-G-AO solution was taken in a 5 mL cartridge and was kept at 4 °C for 2-3 min to allow physical gelation. The cartridge was then loaded into the pneumatic extrusion head of 3D bioprinter. Various single layer patterns and multilayer constructs were printed and crosslinked with UV light for 5 min.

3.14 Stability of 3D printed GelMA-G-AO construct

To study the stability of the 3D printed GelMA-G-AO constructs under physiological conditions, the constructs were crosslinked under UV for 5 min and incubated in MEM complete medium at 37 °C in a CO₂ incubator for seven days. The initial weight (W_i) of the construct (n=4) immediately after crosslinking (day 0) was measured. The final weight (W_f) of the constructs after incubation for days 1, 3 and 7 was also measured. The percentage of weight loss of the construct was calculated using the following equation.

$$\text{Weight loss (\%)} = \frac{W_i - W_f}{W_i} \times 100 \dots\dots\dots \text{Equation 13}$$

3.15 Morphology of 3D printed construct

The surface morphology of 3D printed GelMA-G-AO constructs were analyzed by Scanning Electron Microscopy (SEM, Model: Hitachi, S2400, Japan). Square grid constructs of outer dimensions 10x10x2 mm with internal

perpendicular filaments were 3D printed using extrusion print head of 3D bioprinter (3D Discovery, RegenHU, Switzerland) and crosslinked under UV for 5 min. The samples were then lyophilized, sputter coated with gold (HITACHI, E1010, Japan) and observed under SEM. Images were captured and pore size was measured by image analysis using Fiji Image J software.

3.16 Mechanical property of GelMA and GelMA-G-AO formulation

The mechanical strength of the hydrogels was determined by measuring the compressive modulus by compression test using universal testing machine (UTM) (Bioplus 3345, Instron, USA). The base GelMA formulation was prepared by dissolving 250 mg GelMA in 4.5 mL MEM serum free medium at 37 °C in a water bath. Similarly, GelMA-G-AO was also prepared by dissolving 250 mg GelMA and 150 mg gelatin in 4.195 mL serum free medium. Then 300 µL of Ascorbic acid and 5 µL of α -Tocopherol was added in such a way to obtain a final concentration of 3.4 mM AA and 100 µM α -Toc respectively. 0.5 mL of 5% (w/v) Irgacure 2959 was also added into the GelMA and GelMA-G-AO solution and mixed thoroughly. About 750 µL of each sample were then transferred into 48 well plate and crosslinked under UV light for 5 min to generate cylindrical hydrogels with 10 mm diameter and 10 mm height. The compression test was then performed at 20 °C using a 100 N load of diameter 5 cm. The samples were compressed at a strain rate of 10 mm/min until a strain of 50% was achieved. The compressive modulus of each sample was calculated from the stress-strain curve.

3.17 3D bioprinting of liver tissue with GelMA-G-AO formulation

The main requirement for 3D bioprinting of liver constructs is to develop liver tissues mimicking the native tissue and exhibiting liver-specific functions. Therefore, to generate bioprinted liver tissues with necessary characteristics, bioprinting of liver constructs with bioink GelMA-G-AO formulation comprising of primary rat hepatocytes were carried out.

3.17.1 Isolation of primary rat hepatocytes

Primary rat hepatocytes were isolated from male Wistar rat of 200-250 g body weight with prior approval from the Institutional Animal Ethics Committee (SCT/ABS/IAEC-110/01 dated 04-08-2021). The rats were anesthetized by intramuscular injection of ketamine (10 mg/100 g body weight) and xylazine (1mg/100g body weight). Anesthesia was confirmed by checking the absence of eye blink reflex, pedal reflex, and ear pinch reflex. After anesthetization, intraperitoneal injection of heparin (0.25 mL/100 g body weight) was then administered. The animal was kept in supine position and the abdomen was cleaned thoroughly with 70% ethanol. The rat was placed in a sterile tray inside Biosafety cabinet (ESCO, Labculture Type A2, Singapore) and strapped to hold in position. A V-shaped incision was made on the skin and muscle at the lower abdomen and extend it towards the lateral aspect of the rib cage. The visceral content was moved to the left side of the animal to expose the portal vein. Using a fine tipped curved forceps, two loose ligatures were placed around the portal vein, one ligature was placed just below the right lobe branch of the portal vein and the other just above the pyloric vein. A small incision was made on the portal

vein between the ligatures using pointed scissors. Portal vein was cannulated using a stainless-steel blunt cannula (20 gauge) inserted through the ligature at the distal side. The cannula was secured in position by tightening the ligatures. Pre-oxygenated Calcium-free perfusion buffer (pH 7.4) set at 37 °C was passed through the cannula using a perfusion pump at a flow rate of 20 mL/min. When a color change was observed, turning from deep red to pale yellow, a deep cut was made in the vena cava and perfusion continued at 20mL/min for 10 min. The liver was detached from other organs and the liver was transferred to a stainless-steel sieve and perfused with calcium containing collagenase buffer (0.5 mg/mL Collagenase Type IV Merck-Sigma, cat#.C4-BIOC, Germany) at 30mL/min for 10 min in a recirculation manner. The liver was then disconnected from the cannula and placed in a 150 mm sterile petri dish filled with cold serum-free Iscove's Modified Dulbecco's Medium (IMDM, Merck-Sigma, cat#.56479C, Germany) containing 100 IU/mL of Penicillin and 100 µg/mL of streptomycin. The Glisson's capsule was carefully peeled off and the liver cells were dislodged into the medium by gently shaking. The released cells were passed through a sterile nylon mesh of size 70 µm to remove tissue pieces and cell clumps. The cells were then incubated at 37 °C in CO₂ incubator for a duration of 30 min. The cell suspension was then transferred into a 50mL centrifuge tube and immediately placed on an ice bath for 10min.

Hepatocytes were purified from non-parenchymal cells (NPC) by centrifuging at 50 g for 2 min at 4 °C. The resulting supernatant containing NPCs was discarded while the hepatocyte pellet was washed twice in serum free IMDM with intermittent centrifugation at 20 g for 2 min at 4 °C. The cell viability was

determined by utilizing the trypan blue exclusion method, and only cells with viability exceeding 80% were used for the experiments. The cells were then used within 2 h after isolation or cryopreserved in cryomedium (70% IMDM, 20% FBS, 10% DMSO) and stored at -80 °C until further use.

3.17.2 Optimization of cell concentration in bioink

The cell concentration for liver bioprinting was optimized by standardization of dispensing volume during 3D bioprinting and the concentration of cells in the bioink. The GelMA-AO was prepared by dissolving 500 mg GelMA and 300 mg gelatin in 8.39 mL serum free medium. Then 600 µL of Ascorbic acid and 10 µL of α -Tocopherol was added in such a way to obtain a final concentration of 3.4 mM AA and 100 µM α -Toc respectively. 1 mL of 5% (w/v) Irgacure 2959 was then added into the GelMA-G-AO solution and mixed thoroughly.

The 5 mL GelMA-G-AO solution was transferred to a 5mL cartridge and kept at 4 °C for 3 min. The cartridge was loaded into extrusion print head and the bioprinting parameters were set in the 3D bioprinter. A multilayer construct in grid pattern was 3D printed and the construct was incubated at 37 °C without crosslinking. The constructs got melted and this volume was measured to determine the volume of bioink per construct.

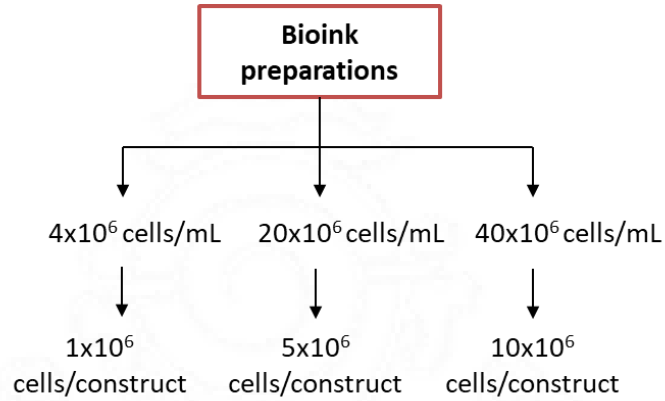


Figure 8. Schematic representation of bioink preparations for obtaining hydrogels containing 1 million, 5 million and 10 million cells/construct.

Bioinks were prepared with three different concentrations of primary rat hepatocytes: 4×10^6 , 20×10^6 , and 40×10^6 cells/mL of GelMA-G-AO solution. 250 μ L of each bioink solutions were transferred into 48 well plate and the time required for each bioinks to undergo crosslinking was noted. The digital images of the encapsulated samples were captured. The encapsulated hydrogels were then transferred into a 24 well plate and cultured in IMDM containing 2% FBS by incubating in a CO₂ incubator at 37 °C. The digital images of the crosslinked hydrogels after incubation for days 1 and 3 was also captured.

3.17.3 Comparison between low and high cell density bioprinting

Liver construct was 3D bioprinted using GelMA-G-AO bioink. Primary rat hepatocytes were isolated from Wistar rat and viability was confirmed by trypan blue dye exclusion staining. The cell suspension was split into two tubes each having total cell number of 4×10^6 cells and 20×10^6 cells respectively. The cell suspension was then centrifuged and medium from each tube was discarded. The GelMA-G-AO solution prepared in IMDM serum free medium was added

into each tube to get bioink of final cell concentration of 4×10^6 cells/mL and 20×10^6 cells/mL. The bioink was transferred into a 5 mL cartridge and kept at 4 °C for attaining physical gelation. A nozzle with an inner diameter of 410 μ m was connected to the cartridge and the cartridge was loaded in the extrusion head of the 3D bioprinter (3D Discovery, RegenHU, Switzerland). The length of the nozzle was calibrated automatically by needle length calibration. The tip of the nozzle was positioned at a height of 200 μ m from the base of the 12 well plate. The extrudability of the bioink was checked by adjusting the pneumatic pressure. The G-code for the design was imported into the RegenHu HMI software, and the printing process was started at a velocity of 10 mm/s. Tissue constructs with 1 million cells/construct and 5 million cells/construct were bioprinted into each well of 12-well plate. The constructs were then exposed to UV for 5 min and 1mL of IMDM containing 2% FBS was added to each well and the constructs were incubated in a CO₂ incubator at 37 °C. The culture media was collected after 24 h for liver specific functional evaluation.

3.17.4 Evaluation of 3D Bioprinted liver

3.17.4.1 *Morphological analysis*

The morphology and distribution of the cells within the bioprinted construct were captured under a phase contrast microscope using a digital camera (VUE model # US300) connected to the inverted microscope ((Nikon Eclipse TS2-FL, Japan).

3.17.4.2 *Liver specific functional evaluation*

Culture media was collected from bioprinted constructs on days 1, 3 and 5 were stored at -80 °C, until used for analyzing albumin and urea secretion.

The albumin secreted by the constructs was quantified using a rat albumin ELISA Quantitation kit (Bethyl Laboratories Inc., Cat no: E111-125, USA) according to the manufacturer's instructions as discussed in the section 3.8.3.1. Albumin standards were prepared as shown in the **Table 5**.

Table 5: Preparation of Albumin standards using Rat reference serum

Standards	ng/mL	Volume of Albumin	Sample dilute
Initial	10,000	4 μ L	12mL
Std 1	125	100 μ L from initial	7.4mL
Std 2	62.5	500 μ L from Std 1	500 μ L
Std 3	31.25	500 μ L from Std 2	500 μ L
Std 4	15.6	500 μ L from Std 3	500 μ L
Std 5	7.8	500 μ L from Std 4	500 μ L
Std 6	3.9	500 μ L from Std 5	500 μ L
Std 7	1.95	500 μ L from Std 6	500 μ L
Std 8	0	-	500 μ L

The amount of albumin synthesized by the construct was determined from a standard curve obtained for rat albumin.

The amount of urea released by the construct was estimated using a commercial kit (Biochain, Cat No: Z5030016, USA) according to the manufacturer's instructions as discussed in the section 3.8.3.2.

3.17.4.3 *Histological analysis*

Cell distribution inside the construct was analyzed by staining paraffin sections with hematoxylin and eosin (H&E). The constructs cultured for 3 days were washed with warm PBS and immersed in 10% Neutral Buffered Formalin and stored at 4 °C until further processing. Subsequently, the fixative was removed, and the constructs were dehydrated using increasing concentrations of isopropanol (70%, 96% and 100%) for 2×30 min. Following this, three xylene washes of 30 min each were performed to clear the isopropanol content. The constructs were then infiltrated with melted paraffin wax for 45 mins and embedded in fresh paraffin wax in a vertical orientation. Thin cross-sections (10 µm) of the embedded constructs were obtained using a microtome (Leica, RM2245, Switzerland) and were mounted on charged glass slides. The sections were stained using the standard hematoxylin and eosin protocol. The stained sections were examined under the bright-field mode of a light microscope (Eclipse TS100, Nikon, Japan), and digital images were captured with a Digital Sight camera (model # DS-Fi2, Nikon).

3.17.5 Functional evaluation of bioprinted liver with high cell density

The culture media was collected from bioprinted constructs on days 1, 3, and 5 were analyzed for albumin and urea secretion. The albumin secreted by the constructs was measured using a rat albumin ELISA Quantitation kit (Bethyl, Cat no: E111-125) according to the manufacturer's instructions. The amount of albumin synthesized by the construct was determined from a standard curve obtained for rat albumin. The amount of urea released by the construct was

estimated using a commercial kit (Biochain, Cat No: Z5030016) according to the manufacturer's instructions.

3.18 Bioprinting of liver construct with modified bioink formulation

3.18.1 GelMA-G-AO bioink with LAP as photoinitiator

The modified bioink formulation was prepared by dissolving 500 mg GelMA and 300 mg gelatin in 6.89 mL serum-free medium at 37 °C. The antioxidants 600 µL of Ascorbic acid and 10 µL of α -Tocopherol were then added from the stock of 56.7 mM AA and 100 mM α -Toc respectively. The final concentration of AA and α -Toc in the bioink was 3.4 mM and 100 µM respectively. A stock concentration of 1% LAP (Lithium phenyl-2,4,6-trimethylbenzoylphosphinate) was prepared by dissolving in PBS at room temperature and from the stock, 2.5 mL of LAP was added to the GelMA-G-AO solution to get a final concentration of 0.25% (w/v) LAP. All the contents were mixed thoroughly by pipetting. The formulation GelMA-G-AO with LAP was denoted as GelMA-G-AO+LAP.

3.18.2 Biological evaluation of GelMA-G-AO+LAP

Cytotoxicity of GelMA-G-AO+LAP was evaluated by direct contact test with L-929 cells based on ISO-10993, Part-5,2009. For the direct contact test, the test sample GelMA-G-AO discs with a diameter of 4 mm and thickness of 2 mm were prepared by cutting them from GelMA-G-AO hydrogel sheets casted on 60 mm petridish. Triplicate samples of GelMA-G-AO hydrogel disc, positive control (PVC) and negative control (UHMWPE) were placed on L-929 monolayer cells

seeded on 24 well plate and incubated at 37 °C for a period of 24 h. The cells surrounding the samples were observed under a microscope, and the cytotoxicity was assessed based on the ISO 10993-5 guideline.

Viability of HepG2 cells encapsulated on GelMA-G-AO+LAP hydrogel was assessed by live/dead staining. A subconfluent culture of HepG2 cells were trypsinized and the cell suspension was centrifuged at 1500 rpm for 2 min and cell pellet at a concentration of 9×10^5 cells was resuspended in 750 μ L of polymer solution by gentle pipetting. HepG2 cells at a density of 3×10^5 cells in 250 μ L of GelMA-G-AO+LAP was transferred into a 24 well plate and was crosslinked under UV for 5 min. The viability of cells encapsulated in GelMA-G-AO+LAP hydrogel was analyzed by FDA/PI staining after culturing in MEM+10% FBS for 24 h. The FDA stock solution was prepared by dissolving 10 mg FDA diacetate (FDA Merck- Sigma, Germany) in 1 mL acetone. 10 μ L of FDA from stock was then added into 1 mL serum free MEM. The cell encapsulated hydrogels were incubated in FDA medium containing 0.01% FDA for a duration of 4 min. 5 μ L of Propidium Iodide (PI, HiMedia, Cat#TC252, India) at a concentration of 0.005% was added to the FDA medium in cells and incubated for 1 min. Hydrogels were rinsed with serum free medium and the cells were then observed under a fluorescence microscope using I3 (green emission for live cells) and N21 (red emission for dead cells) filters.

3.18.3 Analysis of photoprotective property

The photoprotective property of GelMA-G-AO+LAP was evaluated by quantifying the level of intracellular reactive oxygen species (iROS) immediately

after photo-crosslinking using the DCFDA-cellular ROS assay (Abcam ab113851), as previously described. The experiment contained four hydrogel systems: GelMA-G+Irg, GelMA-G-AO+Irg, GelMA-G+LAP and GelMA-G-AO+LAP. The bioink formulations were prepared as shown in the Table 6.

Table 6: Preparation of bioink formulations					
Bioink formulations	GelMA-G solution	AA (56.7 mM)	α-Toc (100 mM)	Irg (5%)	LAP (1%)
GelMA-G+Irg	540 μ L	-	-	60 μ L	-
GelMA-G-AO+Irg	503.4 μ L	36 μ L	0.6 μ L	60 μ L	-
GelMA-G+LAP	450 μ L	-	-	-	150 μ L
GelMA-G-AO+LAP	413.4 μ L	36 μ L	0.6 μ L	-	150 μ L

Four bioink solutions were prepared by mixing a total of DCFDA pre-stained 9×10^5 HepG2 cells with 600 μ L of GelMA-G+Irg, GelMA-G-AO+Irg, GelMA-G+LAP and GelMA-G-AO+LAP. All the bioink formulations were then transferred into a 24 well plate at a volume of 200 μ L/well. The plate was then exposed to UV for 5 min to crosslink bioink and encapsulate the cells within the hydrogels. The constructs were transferred into 1.5 mL vial and HepG2 encapsulated in the four bioink formulations were harvested by incubating the constructs with 200 μ L of 0.3% collagenase – A prepared in calcium containing HEPES buffer for 20 min at 37 °C in water bath. After complete dissolution of hydrogels, the cell suspension was centrifuged at 1500 rpm for 2 min (Eppendorf 5430R, India). The cell pellets obtained were resuspended in 500 μ L PBS and centrifuged at 1500 rpm for 2 min. The PBS was then discarded and the cells were again resuspended in 500 μ L of fresh PBS. The cell suspension was transferred

into a black-clear 96 well plate (Greiner Bio-One). The UV protective property of GelMA-G-AO irrespective of photoinitiators used was analyzed by measuring the fluorescence intensity of 2', 7' – dichlorofluorescein (DCF), which is produced from DCFDA as a result of oxidization by iROS activity, using a multi-well plate reader (Synergy H1, Biotek, USA) at excitation/emission wavelengths of 485 nm/535 nm. The percentage of ROS activity within each GelMA-G-AO formulations (test groups) were determined from the relative fluorescence unit (RFU) of cells retrieved from test groups by normalizing with the base GelMA-G+Irg and GelMA-G+LAP formulations respectively.

3.18.4 UV curing analysis

The crosslinking ability of GelMA-G-AO+LAP was compared with GelMA-G-AO+Irg by UV curing analysis using a rheometer (Physica MCR 301, Anton Paar, Germany) using a parallel plate with geometry of 25 mm diameter and at a measuring distance of 0.5 mm. The UV curing analysis was performed at 20 °C in the oscillatory mode at a constant frequency (10 Hz) and strain (1%) to investigate the photo-polymerization of GelMA-G-AO formulations. Around 0.4 mL of samples were placed between the parallel plates and were exposed to 365 nm UV light source Omnicure 1000 (Lumen Dynamix LDGI) and the variation in storage modulus was recorded up to 200 s.

3.18.5 Compression testing

The mechanical properties of GelMA-G-AO+LAP and GelMA-G-AO+Irg hydrogels were compared by compression test using a universal testing machine

(UTM) (Bioplus 3345, Instron, USA). GelMA-G-AO+LAP and GelMA-G-AO+Irg solutions were prepared as described previously. About 750 μ L of each sample were then transferred into 48 well plate and crosslinked under UV light for 5 min to generate cylindrical hydrogels with 10mm diameter and 10 mm height. The compression test was then performed at 20 °C using a 100 N load of diameter 5 cm. The samples were compressed at a strain rate of 10 mm/min until a strain of 50% was achieved. The compressive modulus of each sample was calculated from the stress-strain curve.

3.18.6 Analysis of spheroid formation by cell encapsulation

Primary hepatocytes at density of 5×10^6 cells were encapsulated in 250 μ L of GelMA-G-AO+Irg and GelMA-G-AO+LAP hydrogel samples. The morphology of the encapsulated cells was observed under phase contrast mode using a digital camera (VUE model # US300) connected to the inverted microscope (Nikon Eclipse TS2-FL, Japan).

3.18.7 3D Bioprinting of liver with GelMA-G-AO+LAP

Hepatocytes were isolated from rat liver as described before. During isolation, the liver suspension obtained was centrifuged at 50 g for 2 min at 4 °C. The supernatant containing non-parenchymal cells (NPCs) were collected and the hepatocyte pellet was resuspended in serum free medium. The hepatocytes were again centrifuged at 20 g for 2 min at 4 °C and the pellet was resuspended in IMDM serum free medium. The collected supernatant containing NPCs were then

centrifuged at 350 g for 3 min at 4 °C and the pellet was resuspended in serum free IMDM. Cell viability was estimated by trypan blue staining.

GelMA-G-AO with LAP formulation was prepared as described before. Then the bioink was prepared by mixing hepatocytes and NPCs at a ratio of $4 \times 10^6 : 1 \times 10^6$ cells in 250 μ L of GelMA-G-AO solution. A design of 10 x 2 mm was prepared with internal filling of perpendicular filaments spaced 1 cm apart. The design was converted to G code so that any 250 μ L bioink will be dispersed for the construct with said dimension. Construct with 5 million cells/construct was bioprinted on 12 well plate using extrusion-based bioprinter. The bioink was crosslinked under UV irradiation at 365 nm. The bioprinted liver tissues were then cultured in IMDM + 2% FBS + antibiotics medium (1 mL/well) in a CO₂ incubator at 37 °C for 48 h. The morphology and distribution of the cells within the bioprinted construct were captured under phase contrast mode using a digital camera (VUE model # US300) connected to the light microscope ((Nikon Eclipse TS2-FL, Japan). The media was removed from the well plate and the constructs were fixed in 10% neutral buffered formalin and stored at 4 °C until further processing. Subsequently, the fixative was removed, and the constructs were dehydrated using increasing concentrations of isopropanol (70%, 96% and 100%) for 2×30 min. Following this, three xylene washes of 30 min each were performed to clear the isopropanol content. The constructs were then infiltrated with melted paraffin wax for 45 min and embedded in fresh paraffin wax in a vertical orientation. Thin cross-sections (10 μ m) of the embedded constructs were obtained using a microtome (Leica, RM2245, Switzerland) and were mounted on charged glass slides. The sections were stained using the standard hematoxylin and eosin

protocol. The stained sections were examined under the bright-field mode of a light microscope (Eclipse TS100, Nikon, Japan), and digital images were captured with a Digital Sight camera (model # DS-Fi2, Nikon).

3.19 In vivo biocompatibility evaluation of 3D Printed construct

3.19.1 Animal ethics

Healthy Wistar rats were received from the Division of Laboratory Animal Science Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (Institution of National Importance, under Govt. of India), Trivandrum. Healthy Wistar rats weighing 200-250g were used for the subcutaneous implantation experiment. All the animals were provided with proper care and maintained at 12 h light and dark cycle maintaining constant temperature of 22 ± 2 °C and relative humidity of 30-70%. Animals were provided with standard pellet diet and water *ad libitum*. In addition, each animal cage was carefully labelled for name, experiment number, number of animals, date of commencement and end of experimental period. Individual animals were marked in the head, neck, body and tail using picric acid for identification. A period of 5 days was provided for acclimatization before the beginning of experiments. All the animals were routinely monitored for health by cage side observation. The animals were handled humanely without causing any pain or stress and with due care for their welfare. All the necessary care and management of the animals were carried out in compliance with the regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Animal experiments were strictly confined to the guidelines of IAEC guidelines which

were approved by the CPCSEA, Govt of India as per the study SCT/IAEC-475/AUG/2023/116, Evaluation of Biocompatibility of materials intended for the fabrication of medical devices: by implantation in rat subcutaneous tissue.

3.19.2 Sample preparation for subcutaneous implantation

3.19.2.1 3D printing of GelMA-G and GelMA-G-AO constructs

GelMA-G and GelMA-G-AO solutions containing LAP as photoinitiator were prepared as previously described. A circular disc shaped multilayer pattern with alternate perpendicular infill having outer dimensions of 10 mm diameter and 2 mm height was designed in 3D BioCAD software. The patterns were printed using GelMA-G and GelMA-G-AO formulation. The printed multilayer constructs were crosslinked and incubated in PBS at 37 °C in a CO₂ incubator for 3 days.

3.19.2.2 Preparation of collagen samples

Lyophilized collagen samples (ColoPlug, India) were cut into circular disc of 10 mm diameter and 2 mm height. The samples were packed in medical grade Eto sterilizer rolls and was sterilized by passing Ethylene Oxide gas (3M Steri-VacTM Sterilizer/Aerator Model 8XL) at 37 °C.

3.19.3 Subcutaneous implantation of 3D printed construct in Rat model

The biocompatibility of the GelMA-G and GelMA-G-AO hydrogels was evaluated by subcutaneous implantation in rat model for a period of 28 days as per ISO 10993-6 Biological Evaluation of Medical Devices”- Tests for local effects after implantation. 3D-printed GelMA-G and GelMA-G-AO constructs of 10 mm

diameter and 2 mm height were considered as test samples, and clinically used lyophilized form of collagen (ColoPlug, India) of a similar size to the test samples was utilized as a control. A total of 10 healthy animals were evenly divided into two groups: 5 rats for the GelMA-G sample and another 5 rats for the GelMA-G-AO sample. Prior to the implantation procedure, the fur on the animals back on both sides of the spinal column were closely clipped. The animals were then anaesthetized by administering intramuscular injections of xylazine (5 mg/kg) and ketamine (100 mg/kg). After 10 min of undisturbed anesthesia, the animals were placed on a surgical table. The skin was thoroughly cleaned with betadine and 70% alcohol. For implanting test samples, two incisions were made on the dorsal surface of each animal, 10 mm apart, on the right side near the spinal column, and then subcutaneous tunnel pockets were created through blunt dissection, and the test samples were inserted into each tunnel. Similarly, on the left side, after creating subcutaneous tunnel pockets, control collagen samples pre-soaked in sterile PBS were implanted. So, in each rat, two test samples and two control samples were implanted on the right and left sides, respectively. The skin incisions were then closed using sterile nylon sutures. The animals were observed at appropriate intervals throughout the experimental period of 28 days for any abnormal findings, including local, systemic, and behavioral abnormalities. At the end of the observation period, the animals were euthanized using overdose of sodium thiopentone. The implantation site along with sufficient surrounding tissue, were collected and fixed in 10% neutral buffered formalin. The fixed tissues were then processed for standard paraffin embedding and 5 μ m thick sections were taken. The sections were mounted on slides and stained with H&E. The stained

sections were examined under the bright-field mode of a light microscope (Eclipse TS100, Nikon, Japan), and digital images were captured with a Digital Sight camera (model # DS-Fi2, Nikon).

3.20 Efficacy evaluation of cell-laden constructs

3.20.1 Animal ethics

All animals were handled humanely, without making pain or distress and with due care for their welfare. The care and management of the animals will comply with the regulations of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Govt. of India. All the animal experiments were carried out after getting prior approval from Institutional Animal Ethics Committee (IAEC) and performed under the approved institutional protocol as per the IAEC approved No: (SCT/ABS/IAEC-110/01 dated 04-08-2021).

3.20.2 Bioprinting of liver constructs

Hepatocytes and NPCs were isolated from rat liver as described before. During isolation, the liver suspension obtained was centrifuged at 50 g for 2 min at 4 °C. The supernatant containing non-parenchymal cells (NPCs) were collected and the hepatocyte pellet was resuspended in serum free medium. The hepatocytes were again centrifuged at 20 g for 2 min at 4 °C and the pellet was resuspended in IMDM serum free medium. The collected supernatant containing NPCs were then centrifuged at 350 g for 3 min at 4 °C and the pellet was resuspended in serum free IMDM. Cell viability was estimated by trypan blue staining. GelMA-G-AO with LAP formulation was prepared as described before. 20×10^6 cells/mL of

bioink was prepared by mixing hepatocytes and NPCs at a ratio of $4 \times 10^6:1 \times 10^6$ cells in 250 μ L of GelMA-G-AO solution. A design of 10 x 2 mm was prepared with internal filling of perpendicular filaments spaced 1 mm apart. Constructs with 5 million cells/construct was bioprinted on 12 well plate using extrusion-based bioprinter. The construct was crosslinked under UV irradiation at 365 nm. Bioprinted liver tissues were then cultured in IMDM + 2% FBS medium in a CO₂ incubator at 37 °C for 48 h.

3.20.3 Transplantation of bioprinted liver into rat model

A total of six healthy male Wistar Rats weighing approximately 200-250g were selected for the *in vivo* transplantation experiments. The rats were anesthetized by intraperitoneal injection of Xylazine (7 mg/kg) and Ketamine (70 mg/kg). The fur on the ventral surface of the animals were clipped and the animals were placed on the surgical table. The skin was thoroughly wiped with 10% betadine solution. An upper midline abdominal incision on skin and muscle was made to access the liver. A disc shaped defect of 10×2 mm size was created in liver using 8mm toothed trephine and the bleeding was controlled by applying gentle pressure with a sterile gauze. When bleeding was subsided or stopped, the bioprinted liver of same size as that of the defect size was placed in the defect. The transplanted liver was covered with the omentum and fibrin glue (Baxter Tisseel Fibrin sealant) was applied around the site of transplantation for fixation. The animals were allowed to recover and were provided with antibiotic Ceftriaxone (25 mg/kg) and analgesic Meloxicam (0.5 mg/kg) for three days. The immunosuppressant drug Tacrolimus at a concentration of 25 mg/mL was

prepared by dissolving 1 mg of tacrolimus in 4 mL normal saline solution. After the surgery, tacrolimus was administered through subcutaneous injection at a dosage of 0.5 mg/kg to prevent graft rejection. Tacrolimus was then provided to the animals for the next five days through drinking water by mixing 1.2 mL of 25 mg/mL tacrolimus with 300 mL of drinking water to provide an intake of dosage of 0.2 mg/kg. The wellbeing of the animals such as food and water intake was monitored every other day for a total of 28 days. The body weight of the rats was recorded regularly. Blood samples of around 0.5-1 mL were collected *via* the retro-orbital route on day 0 (day before surgery) and on days 7, 14, and 28 to assess liver-specific functions. The amount of total protein, albumin, urea, ALT, AST and ALP produced was estimated using Beacon serum kit. On day 28, the animals were euthanized using CO₂ inhalation, and liver tissues from the transplanted site were fixed in 10% neutral buffered formalin. The fixed tissues were then processed for standard paraffin embedding and 5 µm thick sections were taken. The sections were mounted on slides and stained with H&E. The stained sections were examined under the bright-field mode of a light microscope (Eclipse TS100, Nikon, Japan), and digital images were captured with a Digital Sight camera (model # DS-Fi2, Nikon).

3.21 Statistical analysis

The statistical analysis was conducted using the GraphPad Prism software (Version 9, San Diego, USA). All data were presented as the mean plus or minus the standard deviation. Differences between groups were analyzed using Student's t-tests, and a p-value less than 0.05 was considered to be significant.

Chapter 4

RESULTS AND DISCUSSION

GelMA is a photopolymerizable gelatin-based derivatives which is synthesized based on the reaction of free amino groups of lysine/hydroxylysine residues present in gelatin molecules with methacrylic anhydride (Van Den Bulcke *et al.*, 2000). It has been widely utilized in tissue engineering as scaffolds and for 3D bioprinting due its excellent non-cytotoxicity, biodegradability and tunable mechanical strength. Therefore, GelMA was chosen as the biomaterial for bioink formulation in this study.

4.1 Synthesis of Gelatin Methacrylamide

A total of thirteen batches of GelMA were synthesized, and the table shows the percentage yield of GelMA for each batch (Table 7). Approximately 170g of GelMA was synthesized in total with overall yield efficiency of $64.6 \pm 3\%$.

Among the various types of gelatin, porcine gelatin is commonly utilized for tissue engineering applications due to its higher mechanical properties (Shie *et al.*, 2020). Type A gelatin was selected for the synthesis of GelMA as it is less denaturated and hydrolyzed in comparison with type B gelatin. Also, type A gelatin exhibits a closer resemblance to collagen in terms of amino acid composition and isoelectric point (IEP) (Lee *et al.*, 2015). The pH of the reaction mixture must be maintained during GelMA synthesis as the byproduct

(methacrylic acid) can lower the pH, which can hinder the forward reaction by protonating free amino groups. To address this issue, MAA was added sequentially to promote the forward reaction while the pH of the solution was adjusted simultaneously. The carbonate-bicarbonate buffer system used 2-fold of MAA, a much smaller amount of MAA compared to the conventional PBS system which required a molar excess of MAA ranging from 10-32-fold (Shirahama *et al.*, 2016). So, carbonate-bicarbonate buffer could be superior to PBS in terms of rendering free amino groups reactive *via* deprotonation and buffering capacity (Zhu *et al.*, 2019).

Table 7: Yield of GelMA synthesis

Batches	Wt of Gelatin (g)	Wt of GelMA (g)	Yield (%)
GelMA B-1	20	13.6	68
GelMA B-2	20	13.5	67.5
GelMA B-3	20	12.7	63.5
GelMA B-4	20	12.5	62.5
GelMA B-5	20	13.3	66.5
GelMA B-6	20	12.5	62.5
GelMA B-7	20	13	65
GelMA B-8	20	12.7	63.5
GelMA B-9	20	13.6	68
GelMA B-10	20	12	60
GelMA B-11	20	13.5	67.5
GelMA B-12	20	12.5	62.5
GelMA B-13	20	12.7	63.5

4.2 Physicochemical characterization of GelMA

4.2.1 Degree of Functionalization

The degree of functionalization in GelMA was estimated by Ninhydrin assay and Trinitrobenzene Sulfonic acid (TNBSA) assay. The degree of methacrylation of GelMA of batches from 1 to 5 is depicted in Figure 9. The

average value of DoF obtained by Ninhydrin assay and TNBSA assay was $93.6 \pm 3\%$ and $88.8 \pm 5\%$ respectively. The degree of methacrylation is defined as the ratio of the amino groups that are functionalized with methacrylate groups to the total number of amino groups in gelatin before the methacrylation reaction. According to reports, the use of carbonate-bicarbonate buffer at higher pH raises the degree of methacrylation to over 95% (Shirahama *et al.*, 2016). The degree of methacrylation is one of the main factors that influences the biophysiochemical properties of GelMA and its photocured hydrogel (Zhu *et al.*, 2019). It can impact the crosslinking density, porosity, pore size, compressive modulus, swelling and degradation behavior of the hydrogel (Velasco-Rodriguez *et al.*, 2021, Sun *et al.*, 2018). The crosslinking density of GelMA increases as the degree of methacrylation increases, resulting in improved mechanical properties (Suvarnapathaki *et al.*, 2019). In case of bioprinted construct it is necessary to have enough mechanical strength to maintain the shape fidelity. Thus, the result suggests that the in-house synthesized GelMA have a higher degree of functionalization and when used as bioink it can provide adequate shape fidelity and strength to the 3D bioprinted construct after UV crosslinking.

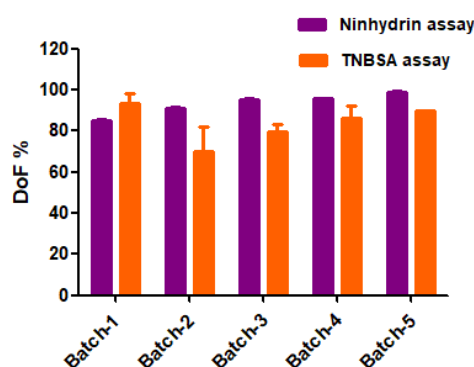


Figure 9. Degree of methacrylation of synthesized GelMA

4.2.2 ^1H Nuclear Magnetic Resonance Spectroscopy

The functionalization of gelatin with the methacrylamide was confirmed by proton NMR. The resonance signals in the NMR spectrum of GelMA at 1.9 ppm corresponded to the methyl group and the peak at 5.4 ppm and 5.7 ppm attributed to the vinyl protons of methacrylamide grafts on hydroxyl lysine and lysine, respectively. The reduced intensity of the lysine signal at $\delta = 2.9$ ppm suggesting modification of lysine by methacrylamide groups.

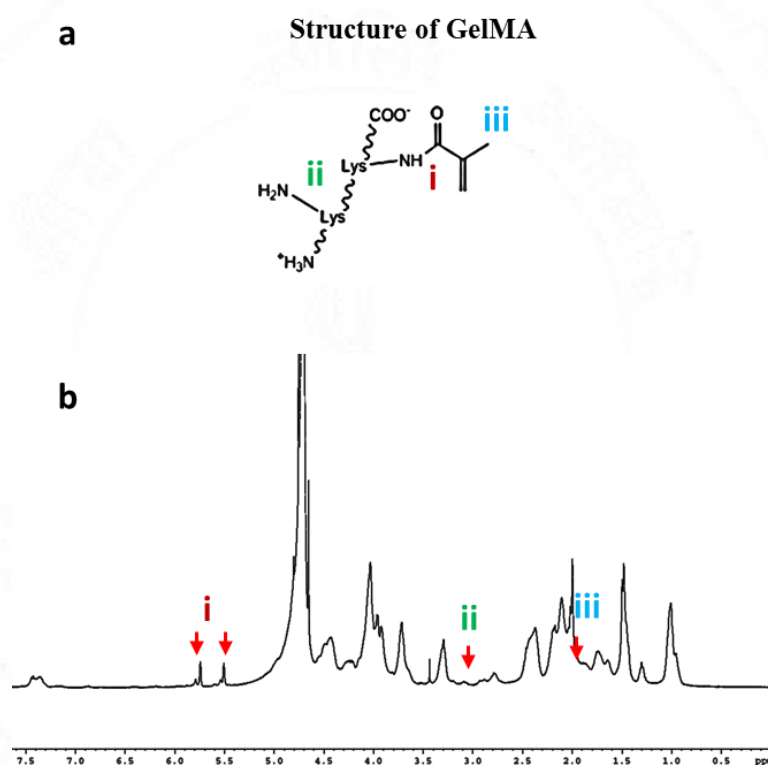


Figure 10. ^1H NMR spectrum of Gelatin Methacrylamide. a) Schematic molecular structure of GelMA showing methacrylated lysine and hydroxyl lysine residues. b) ^1H -NMR spectrum depicting peaks corresponding to vinyl protons of methacrylamide grafts of lysine groups and those of hydroxyl lysine groups (i), lysine methylene (ii) and methyl protons of methacrylamide grafts (iii) in GelMA.

The ^1H NMR spectrum of GelMA matched well with the earlier reports which showed the successful methacrylation of gelatin by the presence of methyl and vinyl proton signals at $\delta = 1.9$ and $5.4/5.7$ ppm, respectively, along with the reduced intensity of the lysine signal at $\delta = 2.9$ ppm (Shie *et al.*, 2020)

4.2.3 Photocrosslinkability of GelMA

The crosslinkability of bioink is essential to stabilize the 3D bioprinted structures. Among different crosslinking mechanisms, photo crosslinking is a commonly used method in 3D bioprinting (Lim *et al.*, 2020).

Researches has highlighted that GelMA hydrogels with low concentrations (≤ 5 w/v%) demonstrate excellent cell viability due to decrease in matrix stiffness, rendering them well-suited for cell-laden bioinks (Yin *et al.*, 2018). At the same time, it is essential to select a bioink concentration that can form crosslinked hydrogels under short crosslinking time as prolonged UV exposure can affect cells. Here, the crosslinkability of different concentrations of GelMA polymer solution (3% ,4% and 5%) was determined by examining the stability and shape fidelity of the hydrogel formed after exposure to UV light. Among the concentrations studied, 3% GelMA was exposed under UV for 7 min but the hydrogel formed exhibited poor shape fidelity. While 4% GelMA solution took 5 min to form crosslinked hydrogel and the 5% GelMA solution, the highest concentration studied, only took 3 min to form hydrogel. Comparing the hydrogels formed by 4% and 5% GelMA, although both were capable of forming hydrogels, 5% GelMA exhibited a more stable hydrogel with superior shape fidelity.

Furthermore, the 5% GelMA hydrogel was mechanically strong enough to be handled with forceps when compared with 4% GelMA hydrogel.

The rheological analysis by frequency sweep test under UV exposure for a period of 200 s showed that the storage modulus of 4% and 5% GelMA was increasing with respect to UV time indicating samples were undergoing crosslinking. The storage modulus of 5% and 4% GelMA crosslinked hydrogels formed after 200 s of UV exposure was 74 Pa and 44 Pa respectively indicating that 5% GelMA formed stronger gel than 4% GelMA. In case of 3% GelMA, no significant increase in storage modulus under UV exposure was noted. Therefore, it shows that 3% GelMA requires more than 200 s of UV exposure time even to initiate the crosslinking process.

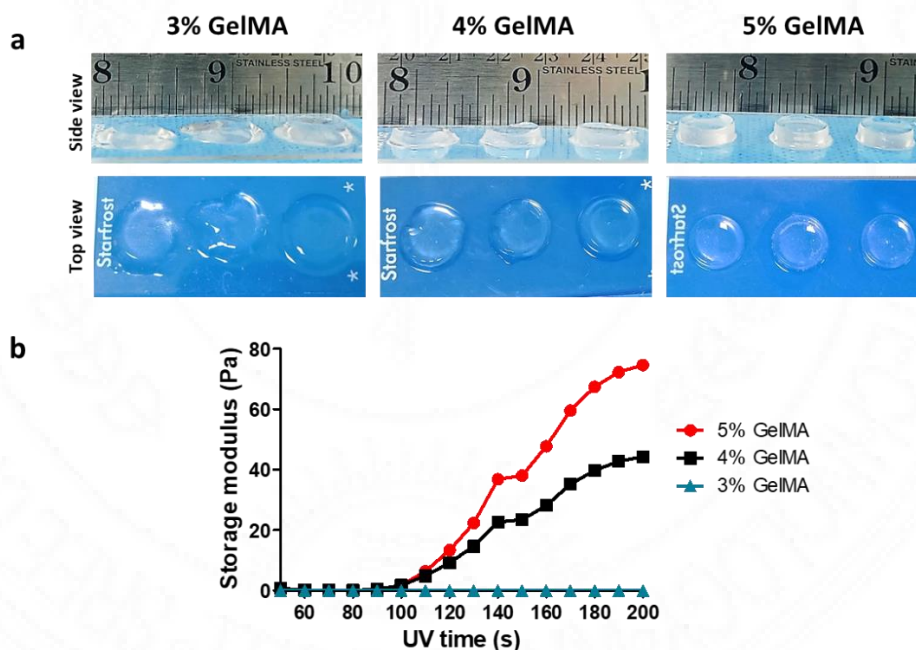


Figure 11. Photo-crosslinking of GelMA. (a) Representative images of UV crosslinked GelMA hydrogels (i) 3 % GelMA, (ii) 4 % GelMA, (iii) 5 % GelMA. (b) Graph depicting the variation of storage modulus of different concentration of GelMA with UV time.

One of the main advantages of GelMA is its tunable property which depends mainly on their degree of substitution, their concentration and selected curing parameters (light intensity, exposure time of irradiation, and amounts of an initiator) (Zhu *et al.*, 2019). The time taken for crosslinking of GelMA depends on the concentration of GelMA and photoinitiator used. Irgacure 2959 is one of the widely used photoinitiators in crosslinking processes under UV irradiation of 365 nm due to its high availability and easy application (Han *et al.*, 2019). About 0.5% (w/v) Irgacure 2959 has been commonly used for the preparation of photocrosslinked hydrogels as it has been reported to be non-cytotoxic (Chan *et al.*, 2010). As concentration of GelMA increases, more methacrylic groups present in the gelatin side chains gets crosslinked and thereby reduces crosslinking time. Therefore, 5% GelMA was selected for further analysis.

4.2.4 Water content of 5% GelMA

GelMA hydrogel at 5% concentration retains 85.3% water on equilibrium swelling at 37 °C. The weight of the lyophilized hydrogel was increased 6.8 times over incubation in PBS. Higher water content of the hydrogel resembles natural tissue environment thereby enhances cell survival, cell growth and proliferation within the hydrogel. Higher water content leads to an increased permeability to nutrients into the hydrogel matrix which important for the cell growth (Komeri *et al.*, 2017).

4.2.5 Stability analysis of 5% GelMA hydrogel

The stability of GelMA hydrogel was evaluated by measuring its weight loss after incubation in PBS at 37 °C for a period of 7 days. The quantitative analysis of weight loss depicted as a function of time is given in Figure 12. An initial weight loss of $19.6 \pm 2\%$ is observed on day 1. The weight loss observed on days 3 and 7 are $25.5 \pm 2\%$ and $28.5 \pm 0.6\%$ respectively indicating no significant change in weight loss from day 3 to day 7.

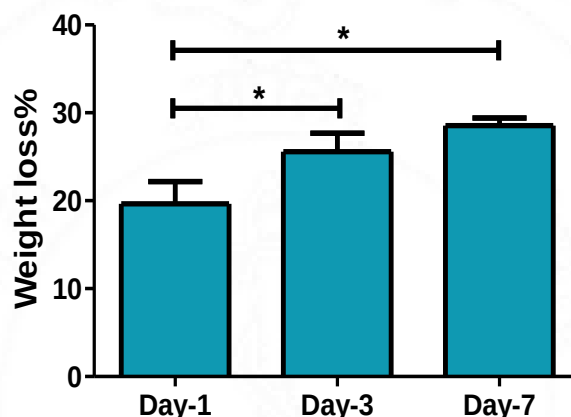


Figure 12. Weight loss of 5% GelMA hydrogel. Graph depicts the weight loss of GelMA over 7 days of incubation in PBS at 37° C. (n=3, *p-value < 0.05)

Weight loss in hydrogels indicates degradation either through hydrolysis or enzymatic mechanisms. Hydrolysis mainly occurs when hydrolytically unstable chemical bonds are present. The rate of hydrolysis depends on crosslinking density, which is depended on polymer concentration or extent of crosslinking during material fabrication (Caliari and Burdick, 2016). Studies have shown that degradation of GelMA hydrogels in PBS is due to hydrolysis (Zhu *et al.*, 2022). As a result, the weight loss that occurred after incubating in PBS under physiological conditions can be attributed to hydrolysis. The results were also

consistent with earlier reports where they observed an initial weight loss of GelMA over incubation in PBS at 37 °C which later became stable (Heltmann-Meyer *et al.*, 2021). It was also observed that the hydrogel maintained its shape despite experiencing weight loss, satisfying the requirement of maintaining the shape fidelity of the printed construct.

4.2.6 Residual EtO analysis and crosslinkability EtO Sterilization

The gas chromatography analysis showed that no EtO residues were detected in EtO sterilized GelMA samples (Figure 13 b). The solubility and crosslinkability of GelMA was remained unaffected after Eto sterilization (Figure 13 c).

GelMA needs to be sterilized prior to cell culture and various sterilization methods are available (Caliari and Burdick, 2016). However, sterilization methods can be destructive, and may have detrimental effects on the naturally-derived hydrogels. So, selecting a reliable and scalable sterilization method for biomaterials used in the bioink is critical for the clinical translation of Bioprinted tissues. According to reports, EtO treatment appears to be the most favorable technique for sterilizing GelMA as this method causes minimal changes in the physical and chemical characteristics of the hydrogels compared to autoclaving and filter sterilization (D O'Connell *et al.*, 2019). But the major concern associated with the ethylene oxide for sterilization arises from its capability to be absorbed by the biomaterials and to react with water or other components, leading to the formation of toxic residues (Rizwan *et al.*, 2020). Therefore, it is important to ensure the absence of Eto residues in the sample after Eto sterilization.

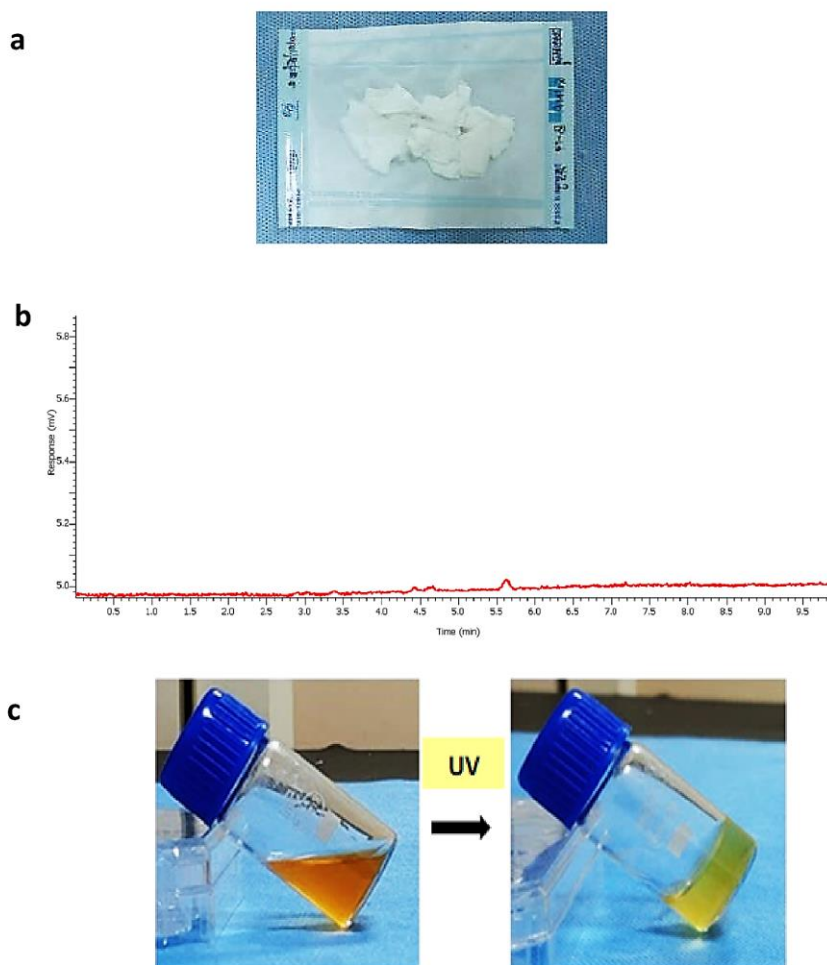


Figure 13. Sterilization of GelMA (a) Eto sterilized GelMA, (b) Chromatogram obtained after residual Eto analysis (c) Solubilized and crosslinked GelMA after Eto sterilization.

4.2.7 Temperature sweep analysis of 5% GelMA

The physical gelation temperature of GelMA was evaluated by studying the effect of temperature on viscosity of 5% GelMA. The gelation temperature was assessed by cooling the polymer solution from 20 °C to 8 °C at a cooling rate of 1 °C/min. It was observed that as the temperature decreased, the viscosity of the

bioink gradually increased. A sudden increase in viscosity was noted when the temperature dropped below 16 °C, indicating sol-gel transition (Figure 14).

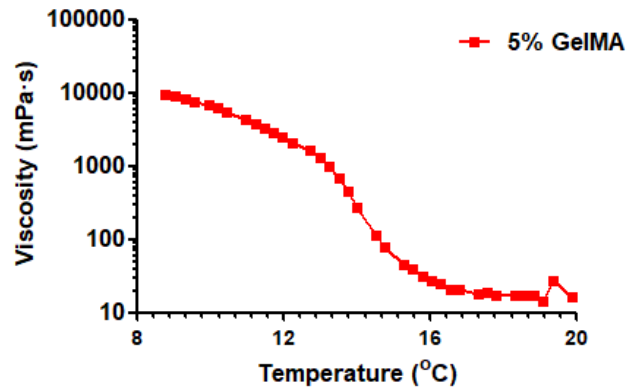


Figure 14. Temperature sweep curve of 5% GelMA. Graph depicts the increase in viscosity of GelMA with respect to change in temperature from 20 - 8 °C.

Sol-gel transition temperature is the temperature at which a solution transforms into gel form. The sol-gel transition temperature of bioink is crucial for setting the printing temperature for ensuring smooth filament extrusion during bioprinting. Temperatures above the sol-gel temperature cause GelMA to liquify, while temperatures below the sol-gel temperature cause over-gelation, and thus affecting printability (Shie *et al.*, 2020). It is also essential for the bioink to be in a gel state during the process of bioprinting in order to have a homogeneous distribution of cells with minimal cell sedimentation within the bioink as cell sedimentation may lead to inconsistent flow rates and nozzle clogging (Jain *et al.*, 2021). It has been reported that low concentrations of GelMA can affect 3D printability because of its poor viscosity leading to formation of non-uniform filaments and delayed gelation, thus affecting the structural integrity of the 3D

printed patterns (Yin *et al.*, 2018). Also, cell viability may be adversely affected at temperatures below 15 °C further highlighting the importance of bioprinting at ambient temperature. According to reports, processability of low-concentration GelMA bioinks was enhanced by blending GelMA with gelatin (Pai *et al.*, 2022a, Yin *et al.*, 2018). In line with this, 3% gelatin was added along with 5% GelMA, and the re-formulated polymer solution was further evaluated.

4.3 Rheological analysis of GelMA-gelatin bioink formulation

The effects of temperature, shear rate, and UV exposure on the flow properties of the 5% GelMA formulation with 3% gelatin was investigated by rheological analysis. A temperature sweep analysis of GelMA-G formulation conducted in the range of 8-24 °C is shown in Figure 15a. The nature of the curve was similar to 5% GelMA and it was observed that the viscosity was inversely proportional to the temperature. The GelMA-G formulation showed a sudden increase in viscosity in the temperature range 20 - 18 °C indicating comparatively higher physical gelation temperature than that of 5% GelMA alone. The outcome was consistent with a previous study that demonstrated the addition of gelatin improves the thermosetting properties of low concentration GelMA, enabling printing at temperatures between 15 and 25 °C (Yin *et al.*, 2018). A semi-viscous condition is required for ideal bioink to form regular filaments and maintain integrity and shape fidelity, while bioink viscosity should be as low as possible to reduce induced shear stress and thus maintain high cell viability (Ouyang *et al.*, 2016). Hence, based on the temperature sweep analysis 20 °C was chosen as the printing temperature and the subsequent rheological analysis were done at 20 °C.

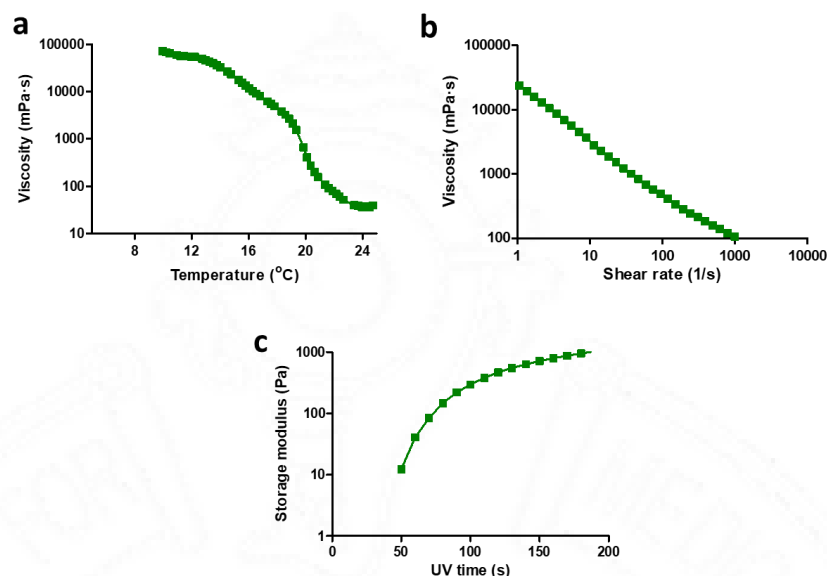


Figure 15. Rheological analysis of GelMA-G formulation. (a) Temperature sweep analysis showing the increase in viscosity of GelMA-G with respect to decrease in temperature, (b) Dynamic viscosity analysis showing the decrease in viscosity as shear rate increases, (c) The increase in storage modulus with respect to UV exposure time.

When extruding the cell-laden bioink, it is important to ensure that the bioink passes through the small orifice of the nozzle without causing excessive shear stress on the cells as it may cause negative impact on cell viability. To achieve this, the bioink should exhibit shear thinning behavior (Holzl *et al.*, 2016). Figure 15b shows the variation of viscosity against the shear rate. The viscosity is seen to linearly decrease with increase in shear indicating the shear-thinning behavior of the GelMA-G formulation. During shear-thinning, the polymer chains are temporarily aligned parallel to the direction of shear which leads to a decrease in viscosity. This decrease in viscosity aids in the formation of smooth filaments at the nozzle. Furthermore, it also aids in maintenance of cell survivability.

The photo-crosslinking analysis under UV irradiation of GelMA-G formulation is shown in Figure 15c. The variation in storage modulus is evaluated against the length of exposure to UV radiation. The storage modulus is found to increase exponentially from 50-200 s indicating that the methacrylamide moieties have been consumed for crosslinking, and the bioink becomes more elastic as the UV exposure time increases, confirming GelMA-G solution undergoes crosslinking.

4.4 Biological evaluation of GelMA-G formulation

4.4.1 Cytotoxicity evaluation

The direct contact test evaluates the impact on cells when they come into contact with a test material. *In vitro* cytotoxicity analysis of the GelMA-G hydrogel based on ISO 10993-5 by direct contact with L-929 fibroblast cells did not show cytotoxic reactivity under or around the sample. Hence, it achieved a score of grade 0, which was considered non-cytotoxic (Figure 16a). The negative control showed non-cytotoxic reactivity and the positive control showed severe cytotoxic reactivity.

The cytotoxicity due to the potential leachant from GelMA-G hydrogel was analyzed by testing the extract method using L-929 cells. After treatment with various dilutions of GelMA-G extract, cells showed non-cytotoxicity with a cytotoxicity grade 0 based on ISO 10993-5. This was comparable to the morphology of cells cultured in the normal medium (cell control) (Figure 16b). Cells treated with diluted phenol showed severe grade 4 cytotoxicity.

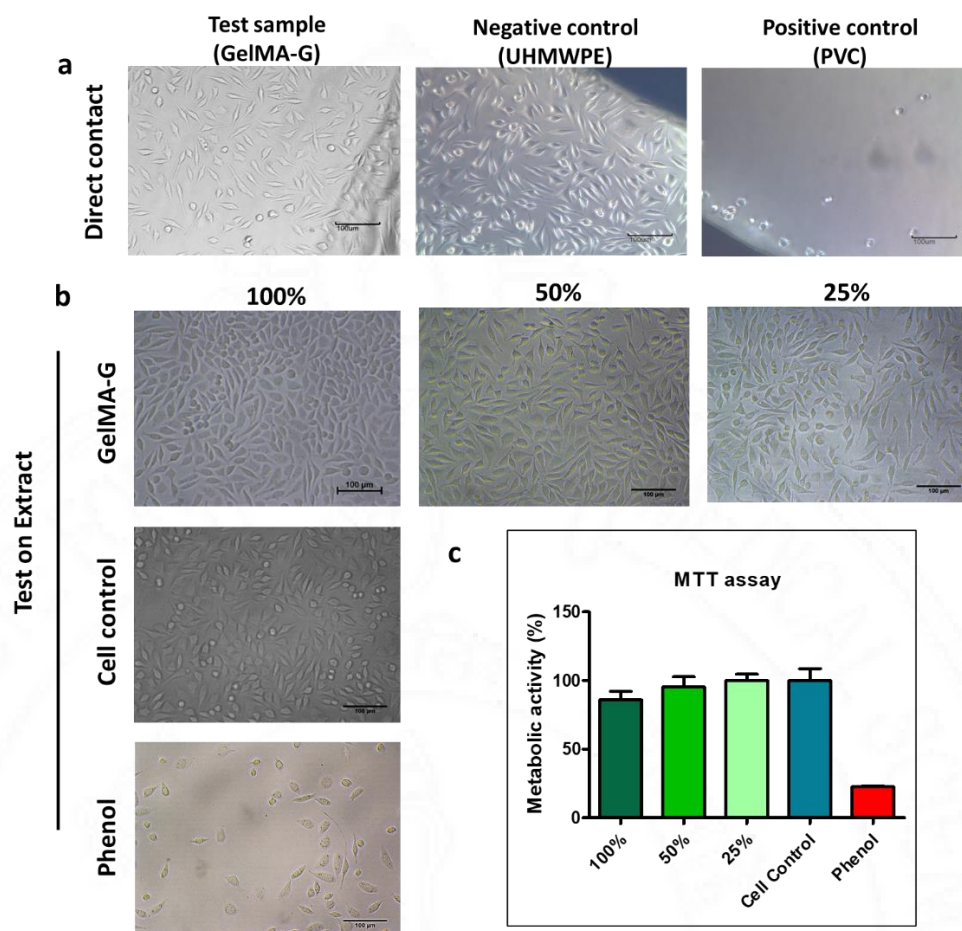


Figure 16. Cytotoxicity analysis of GelMA-G hydrogel. (a) L-929 cells after 24h direct contact with GelMA-G, negative control (UHMWPE) and positive control (PVC). (b) Test on extract analysis showing L-929 cells after 24 h treatment with dilutions of GelMA-G extract, Cell control (culture medium) and phenol. (c) Graph showing cell activity of cells determined by MTT assay. Scale bar 100 μ m

To quantify the cytotoxicity, the MTT assay was performed soon after observation of cells at the end of the treatment time. The activity of cells treated with dilutions of GelMA extract and phenol was normalized to that of the cell control. Cytotoxicity was calculated as a percentage of cell activity (Figure 16c). The 100%, 50%, and 25% extracts of GelMA-G showed mean cell activity of more than 100%, and there was no statistically significant difference compared to cell control. On the other hand, the cells treated with dilute phenol, which is considered

a positive cytotoxic control, showed severe cytotoxicity with a Grade 4 score and < 20 % cell activity.

Hence, this data suggests that there is no potential toxicity due to direct contact or from eluants released from GelMA-G hydrogel. Therefore, GelMA-G can be considered safe for bioink formulation.

4.4.2 Cytocompatibility evaluation

The cytocompatibility of GelMA-G was assessed by evaluating cell adhesion, viability, and proliferation. The staining of cytoskeletal structural proteins in HepG2 cells cultured in GelMA-G hydrogel-coated wells demonstrated that the hydrogel facilitated cell attachment. The cells attained morphology and were evenly distributed on the gel surface (Figure 17a and a').

The viability of HepG2 cells after encapsulation in GelMA-G hydrogel was analyzed using live-dead staining, which revealed that cells were viable immediately after UV crosslinking as well as after culturing for 24 h (Figure 17b and c). The proliferation of HepG2 cells encapsulated in GelMA-G hydrogel analyzed by CCK-8 assay is shown in Figure 17d. The cell activity measured on day 7 after cell encapsulation was normalized to that measured on day 1 and the results show a significant increase in cell activity from day 1 to day 7, supporting evidence for cell proliferation.

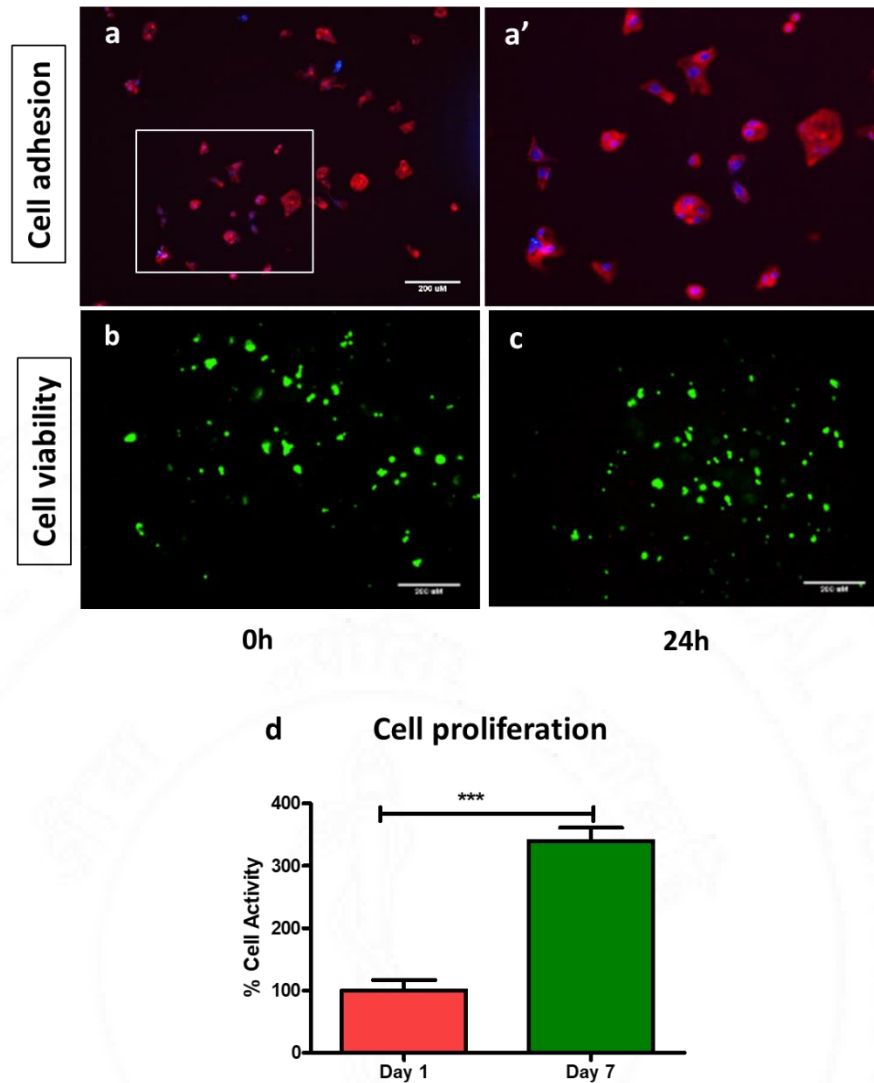


Figure 17. Cytocompatibility of GelMA-G (a) Cytoskeletal staining (red) of HepG2 cells adhered on GelMA-G hydrogel. (a') Magnified area showing cell morphology and spreading. Cell nuclei were counter stained (blue) to identify the cells. HepG2 cells encapsulated in GelMA-G hydrogel showing viability (green) at (b) 0 h and (c) 24 h of encapsulation. (d) Proliferation of HepG2 cells encapsulated in GelMA-G (** $p < 0.0001$). Scale bar 200 μ m

To qualify GelMA-G for formulating bioink for liver bioprinting, it is essential to study its cytocompatibility on liver cells. Hepatoma cell lines are frequently used as an alternative to primary hepatocytes for *in vitro* studies. HepG2 cells are human hepatoma cell line with high proliferation rate and

performs many differentiated hepatic functions (Donato *et al.*, 2015). Therefore, HepG2 cells were used to evaluate the suitability of GelMA hydrogel to support survival and proliferation of encapsulated cells. The result confirms that the hydrogel prepared from the in house synthesized GelMA retained the properties of gelatin such as the presence of cell-attaching and matrix metalloproteinase responsive peptide motifs, which allows cells to proliferate and spread in GelMA-based scaffolds (Yue *et al.*, 2015b).

4.5 Printability assessment of GelMA-G bioink formulation

One of the ideal properties of bioink is printability which indicates that the bioink should be flowable or deformable and be able to be deposited precisely with a good spatial, temporal, and volumetric control (Zhang *et al.*, 2018). Gillispie *et al.* defined printability as “*the ability of a material, when subjected to a certain set of printing conditions, to be printed in a way which results in printing outcomes which are desirable for a given application*”(Gillispie *et al.*, 2020). For extrusion-based 3D bioprinting, printability refers to the suitable extrudability, filament formation and shape fidelity of the extruded single filament as well as the final printed construct as a whole (Schwab *et al.*, 2020). Based on previous reports, a conical shaped printing nozzle having an inner diameter of 410 μm was selected for this study (Ding *et al.*, 2019).

The ability of the bioink to form a continuous filament on extrusion through a nozzle under given printing condition is defined as extrudability and it is the basic requirement for printability (Zhang *et al.*, 2018). So, if a bioink formulation is not strong enough (under gelation), it will result in droplet

formation on extrusion, which will hinder the 3D bioprinting capability. On the other hand, if the bioink is in over gelation condition, it will result in irregular rough filaments. Therefore, it is essential for a bioink formulation to produce a smooth continuous filament upon extrusion for successful bioprinting (Ouyang *et al.*, 2016). The filament extrusion test of the GelMA-G formulation at 20 °C shows the extrudability of the formulation under pneumatic pressure of 35-40 mPa (Figure 18 a). The extruded filament appeared to be smooth and uniform. The results show that the efficient extrudability of GelMA-G formulation is due to the achievement of physical gelation at the printing temperature of 20°C. Also, this ability to form a filament and exhibit uniform extrusion is directly related to the shear-thinning and rapid shear recovery behavior of the bioink (Schwab *et al.*, 2020).

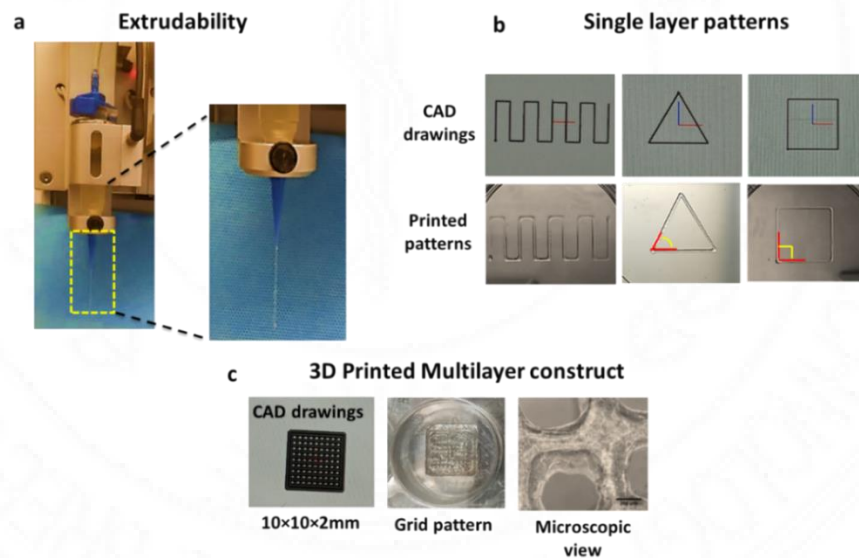


Figure 18. Printability evaluation of GelMA-G (a) Filament extrusion test showing the extruded filament, (b) Single layer patterns: First row represent various designs and second row shows images of the corresponding printed GelMA-G patterns, (c) CAD drawing of multilayer grid pattern and images of the 3D printed GelMA-G construct

The ability to print sharp angles is also considered as one of the methods to evaluate the printability of a bioink. The single layer patterns with various angles were successfully printed using GelMA-G formulation. The 3D printed patterns were accurately aligned with the CAD drawings and there were no instances of filament fusion at the vertices. Image analysis showed that the width of printed filaments was $420 \pm 22\mu\text{m}$ which was closer to the size of the nozzle diameter with no shrinkage or swelling of the filament (Figure 18 b). The 3D printed multilayer square grid construct also demonstrated pattern similar to the CAD drawings (Figure 18 c). The inter-filament spacing in the construct thereby creates a grid pattern which facilitates sufficient mass transfer of nutrients and oxygen to the cells, and also helps in removal of metabolic wastes.

4.6 Stability analysis of 3D printed multilayer grid construct

Polymeric hydrogels have a tendency to exhibit poor long-term fidelity due to their inherent swelling and shrinking properties (Chen *et al.*, 2021). Therefore, it is important to ensure that the printed construct remains stable under physiological conditions. A qualitative analysis of the printed constructs after incubation in culture media at 37 °C revealed that the size of the constructs was reduced while their shape remained unaffected. It has been reported that the gelatin blended along with low concentration of GelMA gradually dissolves when the temperature rises above the gel point of gelatin and this loss of gelatin does not affect the scaffold geometry (Yin *et al.*, 2018).

The % water content in the printed construct analyzed from day 1 to day 7 showed a consistent value of 88%. It has been reported that higher water content promotes hydrolytic degradation of hydrogels (Zustiak and Leach, 2010). Therefore, the reduction in size of the construct might be due to the hydrolytic degradation of GelMA in addition to the release of gelatin from the printed construct. During clinical application, it is essential for the biomaterials to undergo degradation over time to provide adequate time to initiate tissue healing (Amukarimi and Mozafari, 2021). As a result, it is necessary to slow the degradation rate of printed constructs.

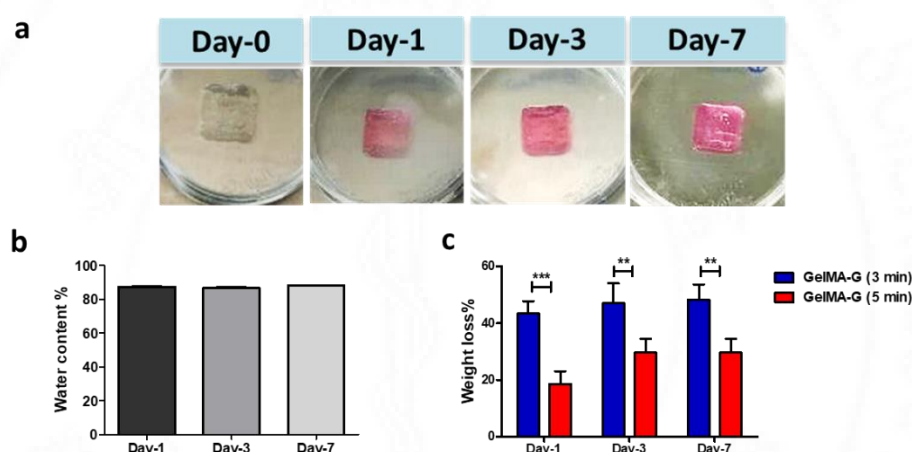


Figure 19. Analysis of multilayer printed grid construct (a) Photographs of the 3D printed GelMA-G constructs after incubated in culture medium for different time points, (b) Graph depicts the water content % in the printed construct, (c) Graph depicts the weight loss (%) of GelMA-G constructs crosslinked for 3 min and 5 min.

The number of crosslinks in the hydrogel network determines the degradation rate of hydrolytically degradable gels. It has been reported that by varying either the macromer concentration, photoinitiator concentration or UV exposure time, the degradation rate can be tailored (Zhao *et al.*, 2020). Therefore,

the stability of the 3D printed construct was further analyzed after increasing UV exposure time from 3-5 min.

The quantitative analysis of weight loss of the 3D printed constructs crosslinked under UV for 3 min and 5 min is shown in Figure 19. The results show that in case of 3D printed constructs crosslinked for 3 min, experienced a decrease of $43.3 \pm 3\%$ on day 1 after being incubated in culture media at 37°C , followed by a further decrease of $46.9 \pm 6\%$ on day 3. Subsequently, no significant weight loss was noted until day 7 ($48.2 \pm 5\%$). On the other hand, when the constructs were stabilized by UV exposure for 5 min, there was a reduction of only $18.6 \pm 3\%$ on day 1, which then reached $29.5 \pm 4\%$ from day 3 and remained consistent until day 7. Therefore, the initial weight loss in constructs crosslinked either for 3 min and 5 min may be attributed to the release of gelatin from the construct, as previously discussed. As a result, by increasing the crosslinking time, the weight loss from the construct due to hydrolytic degradation could be minimized and thereby increases the stability of the construct.

4.7 Hemocompatibility evaluation of GelMA-G construct

Hemocompatibility of blood-contacting biomaterials is an important criterion to be considered for their successful *in vivo* applicability. Hemocompatibility can be estimated by measuring the released hemoglobin into blood plasma upon exposure of whole blood to hydrogels (Li *et al.*, 2013). The percentage hemolysis in plasma after exposure of whole blood to GelMA-G hydrogels was 0.04 %. The percentage hemolysis observed for negative control was 0.03 %. According to ASTM F-756-17 standard, hemolysis up to 5% is

regarded as non-hemolytic and can be considered as blood compatible (Luna-Vázquez-Gómez *et al.*, 2021). Hence, it was demonstrated that GelMA hydrogel exhibit compatibility with blood.

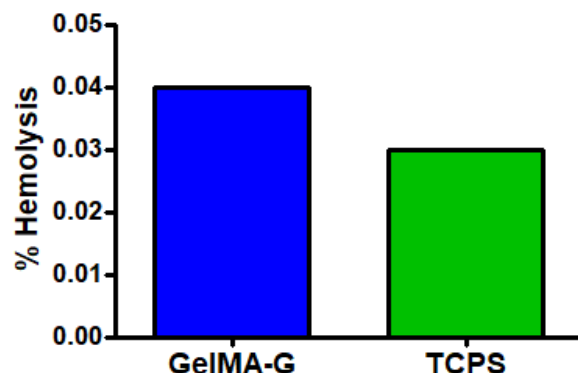


Figure 20. Hemocompatibility of GelMA-G hydrogel. Graph depicts the % haemolysis of GelMA-G and negative control (TCPS).

4.8 3D bioprinting of liver with GelMA-G bioink formulation

Liver tissue constructs containing 6×10^5 HepG2 cells/construct was fabricated using extrusion-based 3D bioprinting. The constructs were accurately aligned with the CAD drawings and maintained the shape fidelity after bioprinting (Figure 21b). The cells were uniformly distributed throughout the construct (Figure 21c) and maintained the cell viability after culturing for 7 days (Figure 21d). HepG2 cells, one of the liver cancers derived cells serves as a suitable cell line for *in vitro* studies of bioprinted liver tissues (Jeon *et al.*, 2017).

Albumin synthesis and urea secretion are important parameters analyzed for evaluating liver specific function of biofabricated tissue. Albumin is the most abundant circulating protein found in plasma secreted by liver. It represents half of the total protein content and acts as the most significant modulator of plasma

oncotic pressure and functions to transport a variety of ligands (Moman *et al.*, 2017). Liver is the only organ that contains the enzymes required for urea synthesis from ammonia detoxification (De Chiara *et al.*, 2018). The functional capability of bioprinted HepG2 construct was monitored for a period of 7 days, and the results showed that the secretion of albumin and urea increased linearly in culture ((Figure 21e and f). Therefore, the results suggest that the GelMA-G bioink formulated from in-house synthesized GelMA qualifies as a suitable bioink for 3D bioprinting liver.

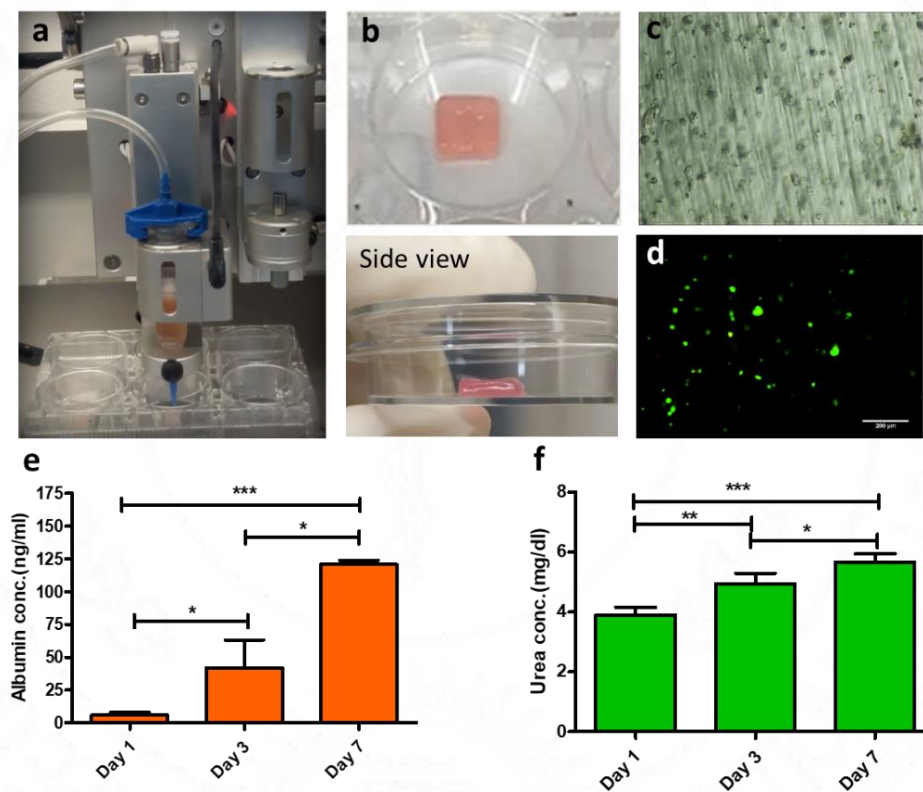


Figure 21. 3D bioprinting with HepG2 cells. (a) Photograph showing pneumatic extrusion of GelMA-G bioink on multi-well plate, (b) Images of top and side view of the Bioprinted liver construct, (c) Phase contrast image showing the distribution of HepG2 cells within the bioprinted construct, (d) Viable cells in bioprinted construct after 7 days of culturing, (e & f) The bioprinted HepG2 construct exhibited linear increase in albumin and urea secretion.

4.9 Phototoxicity analysis in GelMA encapsulated cells

It is well known that UV exposure causes generation of Reactive Oxygen Species (ROS) in cells. One of the commonly used methods to detect ROS is by measuring the fluorescence caused by the conversion of non-fluorescent DCFDA dye, that enters the cell and undergoes conversion to fluorescent DCF in the presence of ROS (Ribeiro *et al.*, 2018). Therefore, to assess the phototoxicity in cells caused by UV, the formation of intracellular Reactive Oxygen Species (iROS) in HepG2 cells during UV exposure was measured.

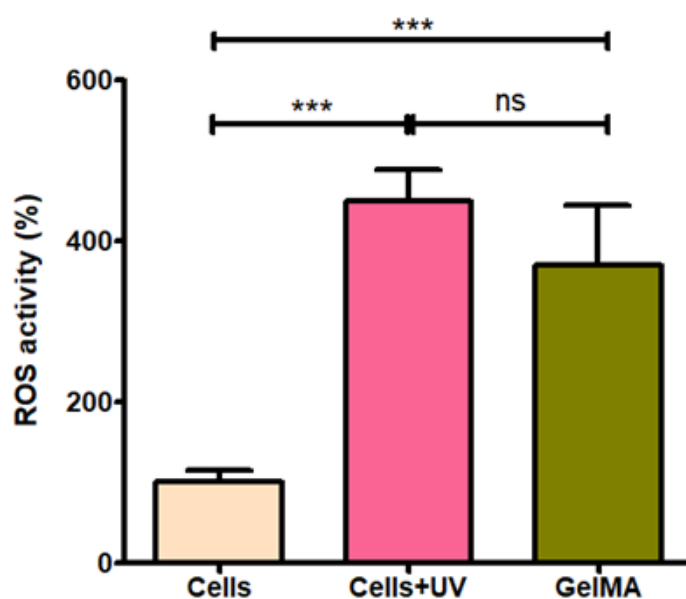


Figure 22. Intracellular ROS production. ROS activity of GelMA encapsulated and UV exposed cells (positive control) were normalized to ROS in normal cells (negative control) that were not exposed to UV. (***) $p < 0.0001$, ns= non-significant). [Reproduced from Anupama Sekar J et al 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

The levels of iROS in HepG2 cells encapsulated in GelMA formulation were compared to normal cells (negative control) and UV-exposed cells (positive

control). The results, shown in Figure 22, indicate that the UV-exposed cells exhibited a $450 \pm 16\%$ increase in ROS production compared to the normal cells. The GelMA encapsulated cells also showed significantly higher ROS activity, reaching upto $370 \pm 30\%$ compared to the negative control.

GelMA is a very well established bioink system for 3D bioprinting of liver tissues as previously discussed. However, there are reports suggesting that the cells can be negatively affected by the free-radical species generated in the presence of photoinitiators during UV exposure (Han *et al.*, 2019). Hence when cells are encapsulated in GelMA-G solution and crosslinked with UV it can also affect the cells. This issue is particularly concerning in the field of bioprinting, where cells are encapsulated within the hydrogel and the presence of radicals raises concerns about the possibility of cytotoxicity. It has also been reported that photoinitiator based systems cause a strong upregulation of reactive oxygen species associated genes, whereas ROS was undetected in radical free photoresins (Rizzo *et al.*, 2023).

Therefore, the detection of iROS level in encapsulated cells demonstrates that a significant amount of free radicals were produced during photo-crosslinking of GelMA. The results were consistent with previous reports which also showed the extensive intracellular ROS production, confirming that UV light has a physiological impact by forming reactive oxygen species inside cells encapsulated in GelMA (Pai *et al.*, 2022a). Thus, it is necessary to develop a GelMA bioink formulation that effectively addresses this limitation. Hence, it was looked deep into the requirement of free radical scavengers in the GelMA based bioink. A synergistically interacting molecules such as Ascorbic acid and α -Tocopherol was

selected to improve the radical scavenging activity and formulate bioink with antioxidant cocktails to make it photoprotective bioink.

4.10 Formulation of GelMA loaded with Antioxidant cocktails

The use of antioxidants as photoprotectants have been extensively documented in the literature. Antioxidants are molecules that are stable enough to donate electron to free radical and neutralize it, thereby reducing its ability to cause damage. These antioxidants delay or prevent cellular damage primarily by scavenging free radicals (Lobo *et al.*, 2010). Non-enzymatic antioxidants such as α -Tocopherol (vitamin E), Ascorbic acid (vitamin C), and carotenoids (vitamin B) are well-known antioxidants in living system. It has been reported that addition of 3.4mM Ascorbic acid in the GelMA bioink can neutralize the excessive ROS generated during UV exposure (Pai *et al.*, 2022a). Furthermore, researchers have shown that combining Ascorbic acid and α -Tocopherol provides better UV protection on skin than either Ascorbic acid or α -Tocopherol acting alone (Lin *et al.*, 2003, Rozman and Gasperlin, 2007). Therefore, Ascorbic acid and α -Tocopherol was loaded into GelMA-G bioink formulation for reducing ROS production and thereby develop a photoprotective bioink.

The concentration of Ascorbic acid utilized for the formulation was 3.4 mM which was a concentration below its IC₅₀ on HepG2 cells as reported by Pai *et al.* (Pai *et al.*, 2022a). In case of α -Toc it has been reported that 100 μ M of α -Toc provides a protective effect against UVAI (340–400 nm)-induced DNA damage in keratinocytes *in vitro* (Delinasios *et al.*, 2018). While finalizing the concentration of α -Tocopherol to be added in the bioink formulation it is important

to incorporate α -toc at a concentration lower than its IC₅₀ because higher concentration can cause cytotoxic effects. Hence, the half inhibitory concentration of α -Toc on HepG2 cells was estimated. It was observed that at 154.4 mM concentration (Figure 23), resulted in 50% loss in viability of HepG2 cells. So, it was decided that the photoprotective concentration of 100 μ M which is previously reported can be used in the formulation of bioink.

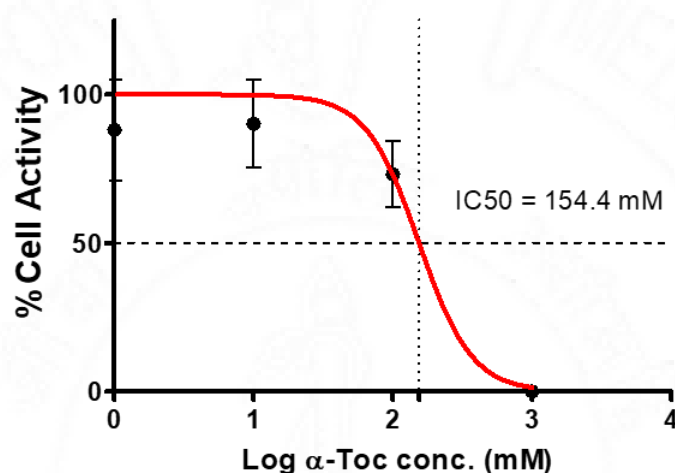


Figure 23. Dose response of α -Tocopherol on HepG2 cells.

Therefore, 5% GelMA+3% gelatin with 3.4 mM Ascorbic acid and 100 μ M α -Toc was formulated as GelMA Antioxidant cocktail bioink (GelMA-G-AO). It is also important to add antioxidants in a way that does not significantly alter the crosslinking time of base GelMA-G bioink formulation (5% GelMA + 3% gelatin). The crosslinking time for the 3D printed construct was optimized at 5 min based on stability analysis (Figure 19). It was observed that the addition of 3.4 mM Ascorbic acid and 100 μ M α -Toc did not significantly affect the crosslinking time of the GelMA formulation.

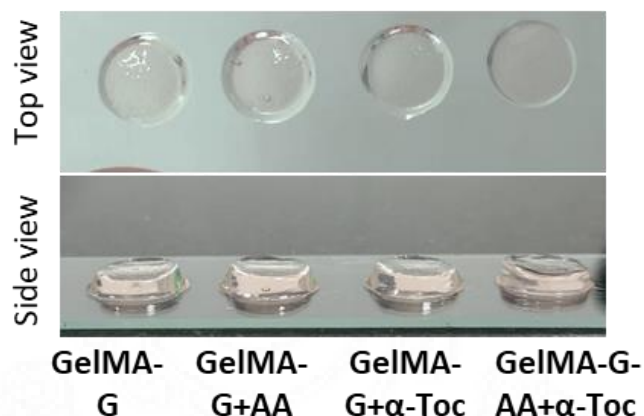


Figure 24. Photocrosslinkability of GelMA-G in presence of antioxidants. Photographs of the crosslinked hydrogels taken after 5 min of UV exposure.

4.10.1 Photoprotective analysis of Antioxidant loaded GelMA-G formulation

The photoprotective effect of GelMA-G bioink formulated with Ascorbic acid and α -Tocopherol was analyzed by measuring iROS production. The result showed that the cells encapsulated in GelMA-G+AA and GelMA-G+ α -Toc produced significantly lower levels of ROS activity ($38 \pm 1\%$ and $57 \pm 3\%$, respectively) compared to those encapsulated in GelMA-G alone. It was also evident from the result that the antioxidant cocktail system, i.e. GelMA-G with a combination of Ascorbic acid and α -Tocopherol (GelMA-G+AA+ α Toc) performed more effectively in providing photoprotection than either antioxidant acting alone, with only $29 \pm 0.4\%$ ROS activity compared to GelMA-G. Therefore, the combination system GelMA-G+AA+ α Toc indicated as GelMA-G-AO (GelMA-Antioxidant) was further studied.

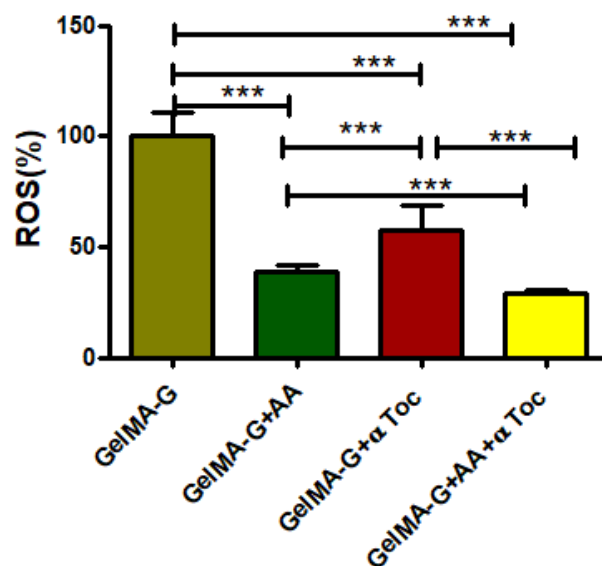


Figure 25. Photoprotective property of antioxidant loaded GelMA formulations. ROS activity in cells encapsulated in GelMA was considered as 100%. ROS activity in antioxidant loaded GelMA hydrogels were compared with GelMA. Combination of AA and α -Toc loaded GelMA showed lesser ROS activity compared to all the formulations individually taken. (***) $p < 0.001$, ns= non-significant). [Reproduced from Anupama Sekar J et al 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

Researches have shown that vitamin E and C work synergistically. When vitamin E scavenges active free radicals, it is converted into vitamin E radical, which could potentially harm polyunsaturated lipids and cause chain oxidation. Whereas in presence of vitamin C, it helps in restoring vitamin E by reducing vitamin E radical, thereby preventing further oxidation caused by the vitamin E radical (Niki, 2010). It is known that ascorbate is an efficient reductant of α -tocopheroxyl radical. This ability of ascorbate to rapidly reduce α -tocopheroxyl radical has been observed in studies using pulse radiolysis and electron paramagnetic resonance (Niki, 2014). Thus, vitamin C helps regenerate the radical form of vitamin E, thereby enhancing its protective capabilities.

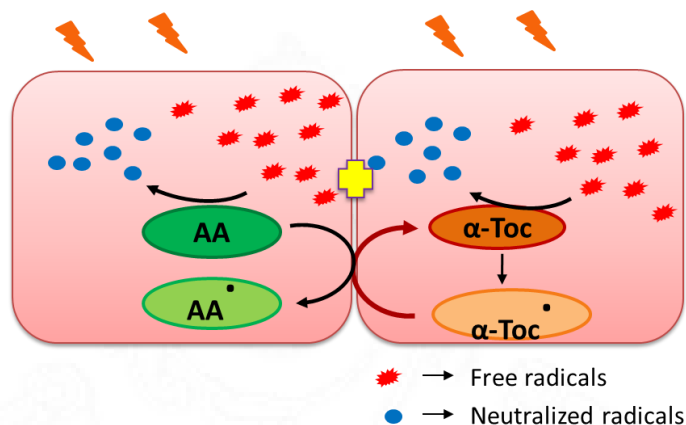


Figure 26. Schematic representation illustrating the synergistic effect of Ascorbic acid and α -Tocopherol incorporated GelMA bioink under UV exposure. In presence of Ascorbic acid, α -Tocopherol radicals generated are converted back into α -Tocopherol.

In the GelMA bioink loaded with antioxidant cocktail system (GelMA-G-AO), vitamin E acts as a scavenger of active free radicals generated during photocrosslinking. As a result, vitamin E is oxidized to form vitamin E radicals. In the presence of vitamin C, vitamin E radicals are then converted back to vitamin E, aiding in the elimination of additional free radicals. The synergistic interaction between Ascorbic acid and α -Tocopherol was evident in the outcome that resulted in enhanced photoprotection when used in combination.

4.10.2 Cell viability

The viability of HepG2 cells encapsulated in GelMA-G-AO hydrogel showed significantly higher viability after 24h of culturing when compared with GelMA-G encapsulated cells. It is well known that oxidative stress affects cell viability and the inclusion of antioxidants in the GelMA-G formulation reduced oxidative stress and thereby improved cell survival.

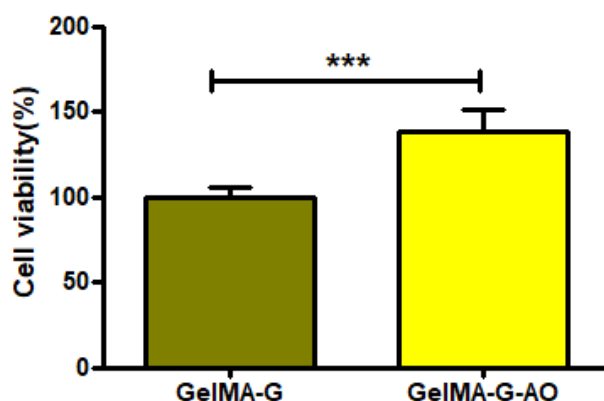


Figure 27. Viability analysis of GelMA-G and GelMA-G-AO encapsulated cells (*** $p < 0.0001$).

Studies have shown that cells cryopreserved with α -Tocopherol showed increased cell viability due to the reduction of ROS production (Aliakbari *et al.*, 2017). Similarly, the effectiveness of Ascorbic acid in neutralizing free radicals and minimizing the negative effects of photoinitiators was studied and the results indicated that treatment with Ascorbic acid had a substantial positive impact on cell viability (Sabnis *et al.*, 2009). In another study it was reported that the combination treatment of α -Tocopherol and Ascorbic acid increases viability of PC12 cells from melamine induced injury through the downregulation of oxidative stress (An *et al.*, 2014).

Therefore, the photoprotective GelMA based bioink formulation GelMA-G-AO was further evaluated.

4.11 Biological evaluation of GelMA-G-AO hydrogel

4.11.1 Cytotoxicity evaluation of GelMA-G-AO hydrogel

To determine whether direct contact with antioxidant-loaded hydrogel or leachants from the hydrogel has any toxic effect on cells, an *in vitro* cytotoxicity analysis of the GelMA-G-AO hydrogel based on ISO 10993-5 was performed on L-929 cells. From the direct contact test, it was observed that cells had no cytotoxic reactivity under or around the hydrogel indicating GelMA-G-AO is non-cytotoxic.

Similarly, the cytotoxicity due to the potential leachants from GelMA-G-AO hydrogel analyzed by test on extract method also showed that cells did not show change in morphology when compared with untreated cells. The MTT assay followed by treatment with extracts revealed that 100%, 50%, and 25% GelMA-G-AO extracts had mean cell activity levels greater than 100%, with no statistically significant difference from the cell control (Figure 28 b). Therefore, it was confirmed that leachants from the GelMA-G-AO hydrogel also did not cause cytotoxicity.

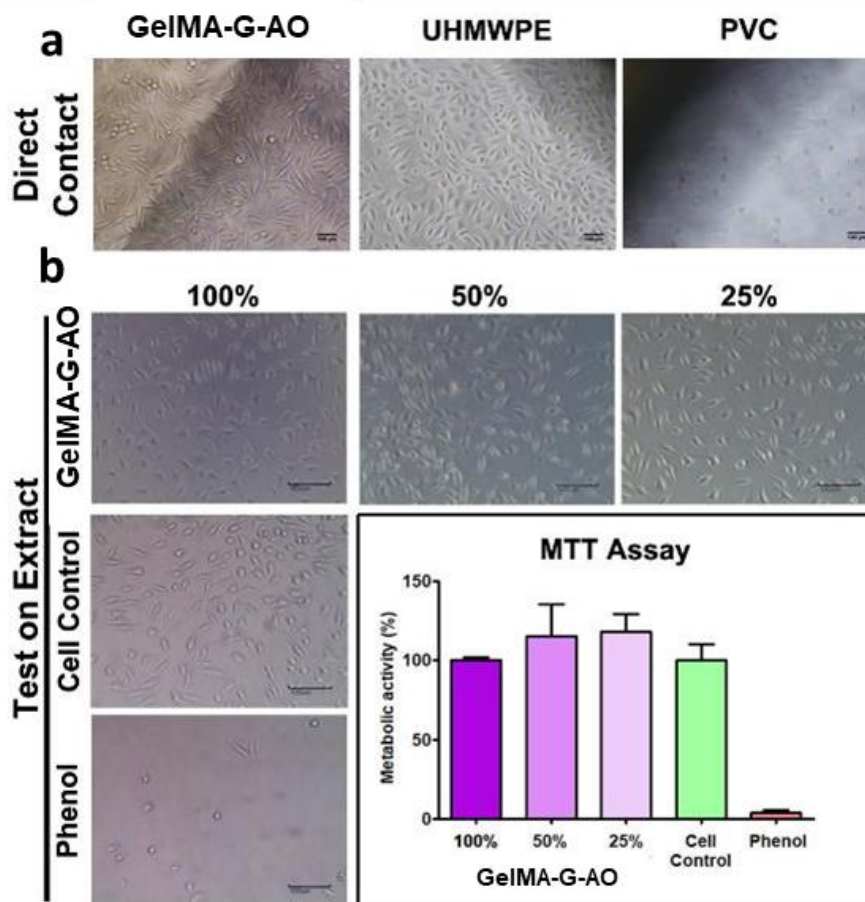


Figure 28. Cytotoxicity analysis of GelMA-G-AO hydrogel. (a) L-929 cells after 24h direct contact with GelMA-G-AO, negative control and positive control. (b) Test on extract analysis showing L-929 cells after 24 h treatment with dilutions of GelMA-G-AO extract, Cell control and dilute phenol. (c) Graph showing cell activity of cells determined by MTT assay at the end of Test on Extract. [Reproduced from Anupama Sekar J *et al* 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

4.11.2 Cytocompatibility evaluation of GelMA-G-AO hydrogel

Cell adhesion studies showed that GelMA-G-AO hydrogel facilitated HepG2 cell attachment on hydrogel coated wells enabling the cells to attain morphology (Figure 29a and a').

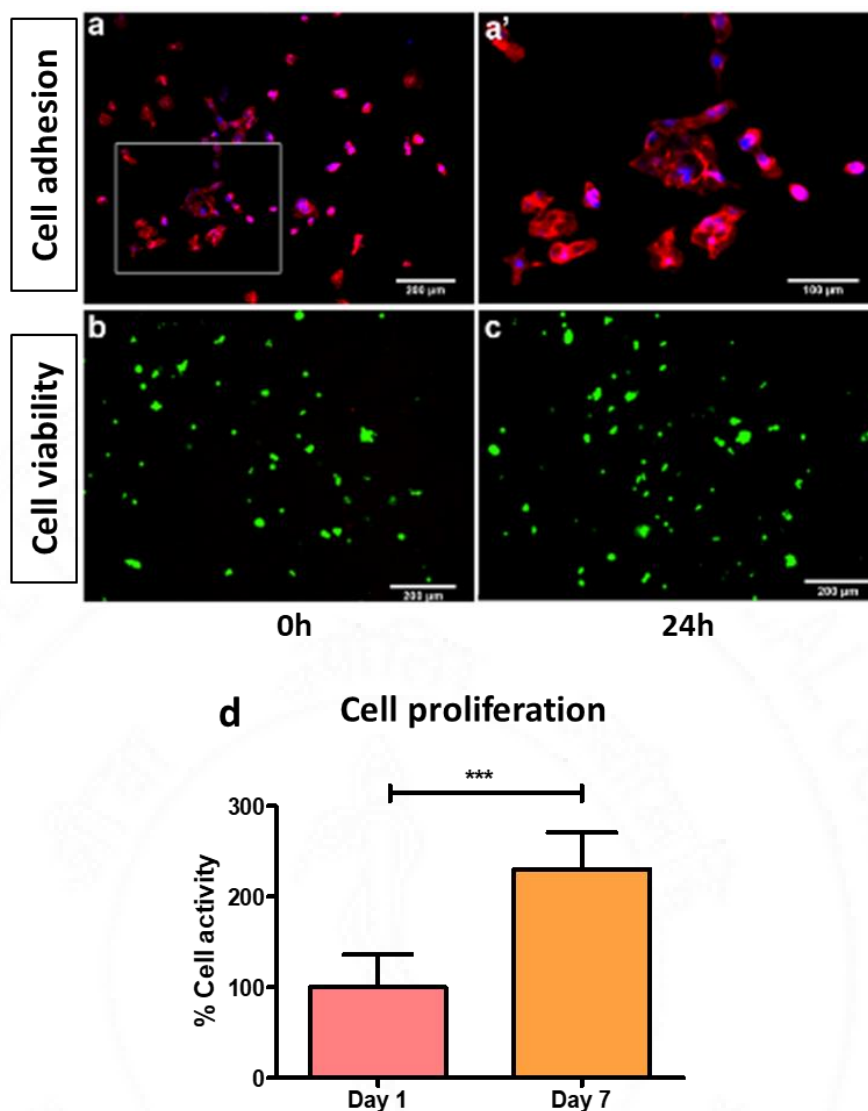


Figure 29. Cytocompatibility of GelMA-G-AO (a) Cytoskeletal staining (red) of HepG2 cells adhered on GelMA-G-AO hydrogel. (a') Magnified area showing cell morphology and spreading. Cell nuclei were counter stained (blue) to identify the cells. HepG2 cells encapsulated in GelMA hydrogel showing viability (green) at (b) 0 h and (c) 24 h of encapsulation. (d) Proliferation of HepG2 cells encapsulated in GelMA-G-AO (*** $p < 0.0001$). Scale bar 200 μ m [Reproduced from Anupama Sekar J *et al* 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

The viability of HepG2 cells encapsulated in the GelMA-G-AO hydrogel showed that cells were viable immediately after crosslinking and after 24h of culturing (Figure 29b). The proliferation of HepG2 cells encapsulated in the

GelMA-G-AO hydrogel analyzed by the CCK-8 assay is shown in Figure 29d. The measurement of cell activity on the 7th day following cell encapsulation, when compared to the measurement on day 1, revealed a notable rise in cell activity from day 1 to day 7, which could potentially be attributed to cell proliferation.

The properties of GelMA which makes them cytocompatible was not significantly affected by the addition of antioxidants to the base GelMA formulation and therefore confirms that GelMA-G-AO hydrogel qualifies one of the ideal properties of bioink.

4.12 Rheological properties of GelMA-G-AO bioink formulation

The rheological properties of GelMA-G-AO formulation with regard to temperature, shear rate and UV exposure time was assessed to determine whether addition of antioxidants has caused significant changes in the critical rheological characteristics of base GelMA-G bioink formulation. A temperature sweep analysis of GelMA-G-AO formulation conducted in the range of 8-24 °C is shown in Figure 30a. Temperature sweep analysis revealed the thermoresponsive nature of GelMA-G-AO formulation, with a sol-gel transition temperature at 18-20 °C, allowing for 3D bioprinting at this temperature. The nature of the curve was similar to that observed in GelMA-G formulation (Figure 15a).

The shear-thinning property of the GelMA-G-AO formulation which is necessary for formation of smooth filaments during extrusion as well as for maintenance of cell survival was also evident from the viscosity curve shown in Figure 30b. The viscosity is seen to linearly decrease with increase in shear, indicating the shear-thinning behavior of the GelMA-G-AO formulation.

The addition of antioxidants may hamper crosslinking ability (Sabnis *et al.*, 2009). Dynamic crosslinking analysis revealed an increase in the storage modulus with respect to increasing UV exposure time from 50-200 s is shown in Figure 30c. The storage modulus is found to increase exponentially from 50-200 s indicating that the GelMA-G-AO solution undergoes crosslinking. The results were consistent with rheological data of base GelMA-G bioink formulation which confirms that the addition of antioxidants did not affect gelation temperature, shear-thinning behavior and photocrosslinkability of base GelMA-G bioink formulation.

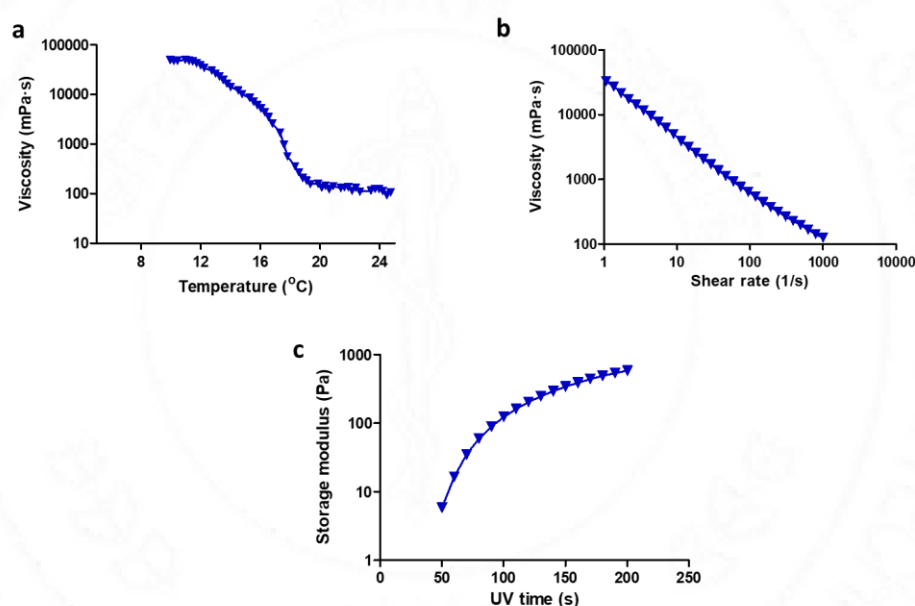


Figure 30. Rheological behaviour of GelMA-G-AO bioink formulation (a) Temperature sweep analysis showing the increase in viscosity of GelMA-G-AO with respect to decrease in temperature, (b) Dynamic viscosity analysis showing the decrease in viscosity as shear rate increases. (c) The increase in storage modulus with respect to UV exposure time. [Reproduced from Anupama Sekar J *et al* 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

4.13 Printability evaluation of GelMA-G-AO bioink formulation

The printability of GelMA-G-AO formulation was evaluated by printing single layer patterns and multilayer constructs. Single layer patterns with various angles were printed and the patterns were accurately aligned with the CAD drawings and there were no instances of filament fusion at the vertices. The multilayer square grid constructs with different dimensions were also successfully printed using GelMA-G-AO formulation (Figure 31a).

The 3D printed multilayer GelMA-G-AO construct was examined using SEM analysis. The analysis demonstrated the grid structure of the construct and also revealed its filamentous and porous characteristics, which included both macro and micro pores (Figure 31 b). The average pore size measured by image analysis was $102 \pm 20 \mu\text{m}$. The pore size and porosity of scaffolds are important for the diffusion of growth factors and the development of blood vessels, which helps to maintain liver functions. Hepatocytes consume significantly more oxygen than other cells, so the size of the pores in the scaffold is a crucial factor for the transfer of oxygen and nutrients to support cell growth. It has been reported that porous chitosan scaffolds with pore sizes ranging from 50 to 150 μm provide ideal conditions for hepatocyte culture (Wu *et al.*, 2007). Also, for liver tissue engineering, which involves a high density of cells, it has been noted that a macroporous structure with pore sizes between 100-500 μm is needed for high-density cell seeding and efficient supply of nutrients and oxygen to the seeded cells (Park and Biomaterials, 2002).

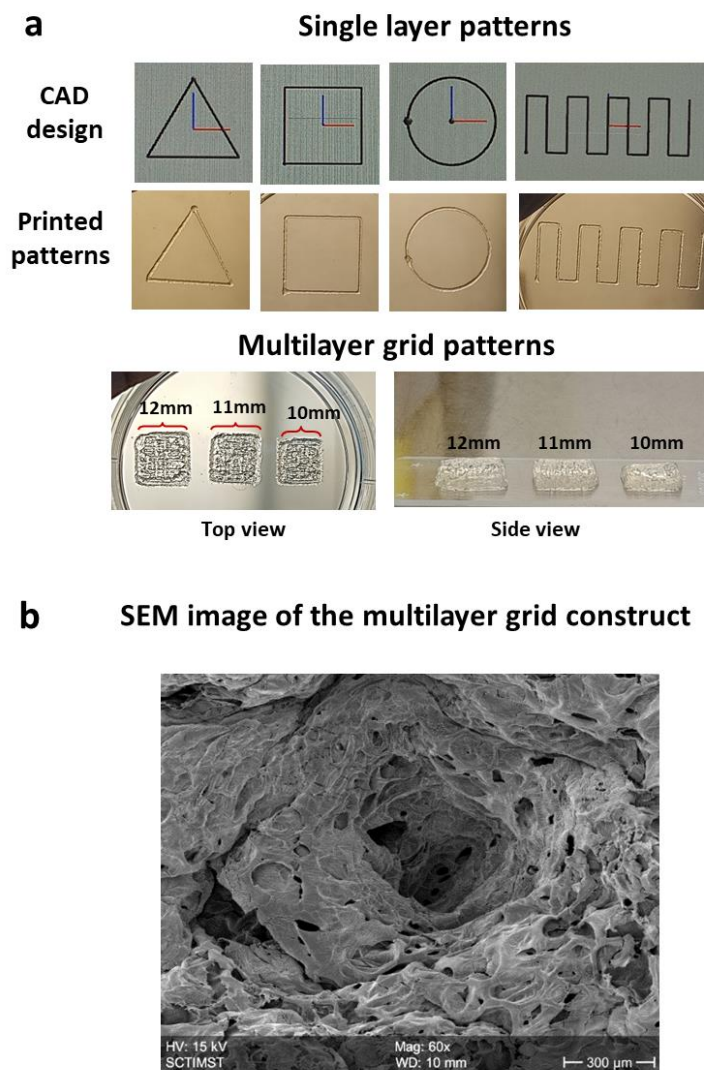


Figure 31. 3D Printing of constructs with GelMA-G-AO formulation (a) Printed patterns with GelMA-G-AO, (b) SEM image of GelMA-G-AO construct showing porous nature [Reproduced from Anupama Sekar *J et al* 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

The stability of the 3D printed GelMA-G-AO construct under physiological conditions was analyzed based on weight loss over time. The constructs were stable in culture medium at 37 °C and maintained their shape over a period of 7 days (Figure 32). The quantitative analysis of weight loss shows that there was a slight reduction of $12.6 \pm 6\%$ on day 1 and, reaching up to $20.2 \pm 6\%$

on day 3. On subsequent days, there were no further weight loss observed until day 7 ($20.7 \pm 6\%$) and the construct maintained $79.2 \pm 7\%$ weight that of the original weight after printed.

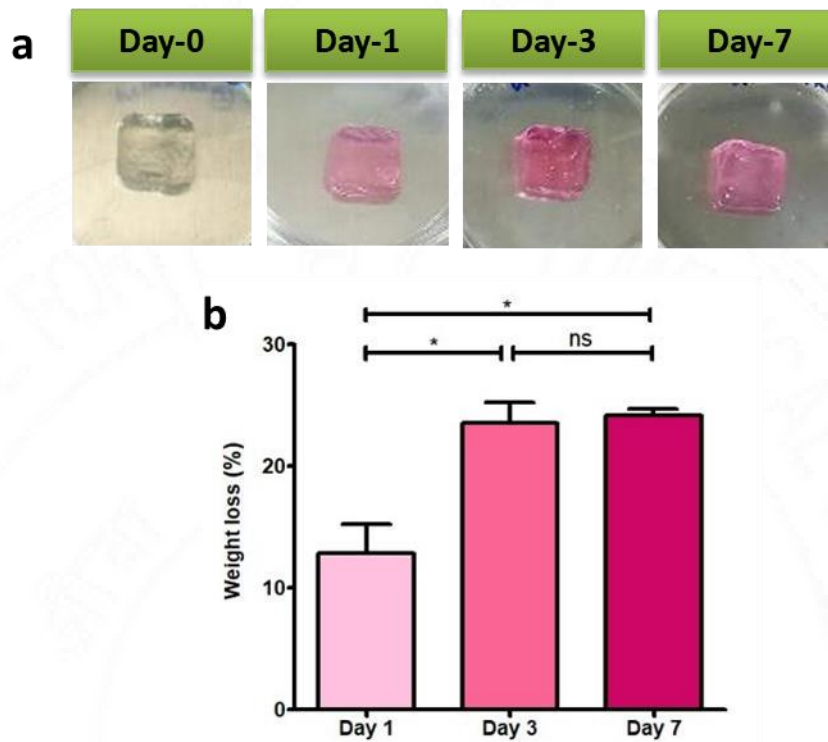


Figure 32. Stability analysis of 3D printed GelMA-G-AO construct. (a) Images of the 3D printed construct taken on different time points,(b) Graph depicts the percentage weight loss of 3D printed constructs on day 1, 3 and 7. [Reproduced from Anupama Sekar J et al 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

Another important factor to be considered is the stability of the construct during *in vitro* maintenance at 37 °C in MEM culture medium. The stability of the GelMA-G-AO construct after crosslinking for 5 min showed a similar trend as observed in GelMA-G construct crosslinked for 5 min. Therefore, the weight loss observed during the initial days could be attributed to the release of gelatin from the construct and the release of gelatin has no significant effect on the shape or

size of the constructs as previously discussed. The results show that the addition of antioxidants to the bioink did not cause significant effect on the stability of the printed construct.

4.14 Mechanical property

The compressive modulus of a hydrogel is a measure of its mechanical strength or stiffness. It has been reported that the rigidity of ECM or the hydrogel stiffness has a significant impact on cell behavior, proliferation, migration and function. Studies have reported that hydrogel scaffolds with stiffness in the range 4-10 kPa promoted better maintenance of hepatocyte phenotype whereas stiffer hydrogels (> 20 kPa) facilitated epithelial - to - mesenchymal transition and induce apoptosis (You *et al.*, 2013, Xia *et al.*, 2020). The average compressive modulus of 5% GelMA alone and 5% GelMA+3% gelatin + AO were 6.3 ± 0.5 kPa and 5.5 ± 0.7 kPa, respectively (Figure 33). This confirms that the mechanical strength of GelMA remained unaffected by the addition of gelatin and antioxidants.

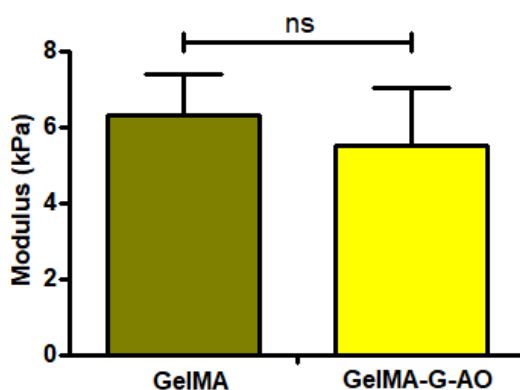


Figure 33. Compressive modulus. The graph shows that there is no significant difference in compressive modulus between GelMA and GelMA-G-AO.

4.15 Three-dimensional bioprinting of liver construct with GelMA-G-AO bioink

The main requirement for 3D bioprinting of liver constructs is the fabrication of liver tissue that mimics the native tissue and exhibits liver-specific functions. The liver tissue is made up of 70% parenchymal cells, 30% non-parenchymal cells, and fewer ECM components. The ECM constitutes a small portion, comprising less than 3% of the total area, within a normal liver section (Gordillo *et al.*, 2015). Thus, liver has a high cellular density (1.3×10^8 cells per gram of tissue) and it is critical to achieve comparable densities during the bioprinting process in order to accurately replicate the functional and physiological aspects of native tissue (Leberfinger *et al.*, 2019). Also, it has been reported that maintaining high cell density within porous scaffolds is very important to induce cell–cell interaction and communication, and thereby promote hepatocyte function (Park and Biomaterials, 2002).

4.15.1 Optimization of cell concentration in bioink

As a preliminary step towards bioprinting of liver tissue with high cell density, it is necessary to optimize the cell concentration in the bioink formulation. Hepatocytes isolated from rat liver having viability >80% were used for bioink preparation. The volume of bioink required for 3D printing multilayer grid construct of size 10×10×2 mm was approximately 250 µL. Therefore, to achieve the optimal cell concentration per construct, an initial study was conducted by encapsulating cells at 1×10^6 , 5×10^6 and 10×10^6 per 250 µL of hydrogel. The optimal crosslinking time for the 3D printed construct was determined to be 5 min

through stability analysis. Cell encapsulation analysis showed that within the same time frame, stable hydrogels with shape fidelity were formed when using both 1×10^6 and 5×10^6 cells per gel (Figure 34a).

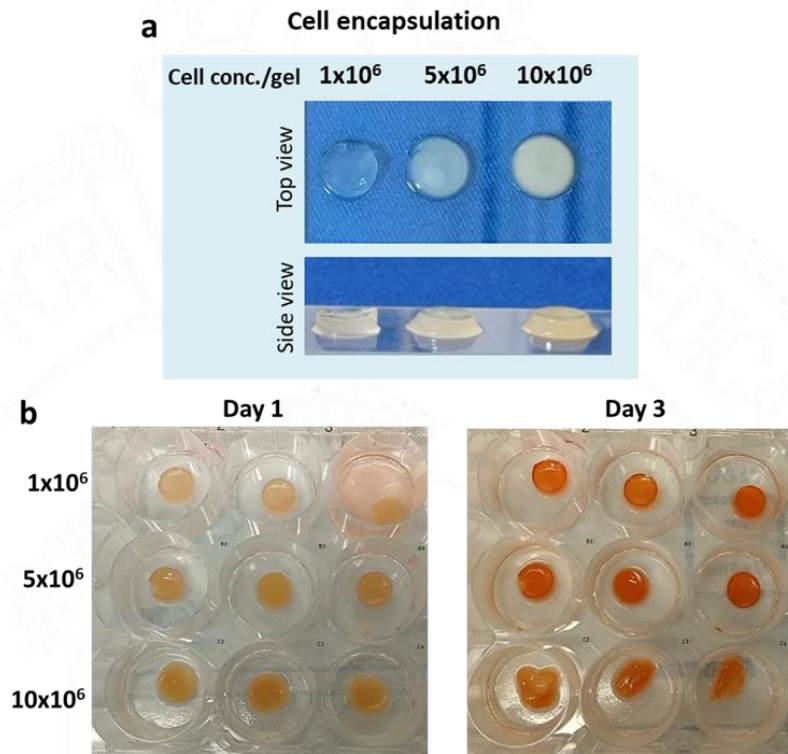


Figure 34. Encapsulation of hepatocytes at various cell concentrations.(a) Crosslinked hydrogels containing cells with varying concentrations, (b) Photographs of the cells encapsulated hydrogels taken after incubation in culture medium on days 1 and 3.

However, a higher cell concentration of 10×10^6 cells per gel required approximately 7 min to form a stable hydrogel. It was further observed that all the crosslinked gels with different cell concentrations remained stable after culturing for 24 h. However, the hydrogel containing 10×10^6 cells experienced deterioration of gel integrity after 72 h of culturing in IMDM culture media at 37°C , which resulted in the release of the encapsulated cells (Figure 34b). Therefore, bioink formulation with 4×10^6 cells/mL and 20×10^6 cells/mL concentrations of rat

hepatocytes which results in cell concentrations 1×10^6 and 5×10^6 per hydrogel were selected for further 3D Bioprinting studies.

4.15.2 Comparison between Bioprinted liver with 1M and 5M cells/construct

The cell number for high cell density bioprinting was optimized at 4×10^6 cells/mL and 20×10^6 cells/mL concentrations of rat hepatocytes. 3D Bioprinted liver construct with 1×10^6 cells (1M) and 5×10^6 cells (5M) per construct is shown in Figure 35a and b. Liver-specific functions in the 3D bioprinted constructs are analyzed by measuring albumin and urea synthesis. Figure 35c demonstrates a significant increase in albumin and urea secretion in the 5M cells/construct compared to the 1M cells/construct. Albumin secretion in 5M constructs (281 ± 20 ng/mL) was approximately 5 times higher than in 1M constructs (53 ± 7.2 ng/mL), indicating sustained protein synthesis in high cell density constructs. Urea secretion in 5M constructs (5 ± 0.2 mg/dL) was 2.5 times higher than in 1M constructs (2.2 ± 0.05 mg/dL).

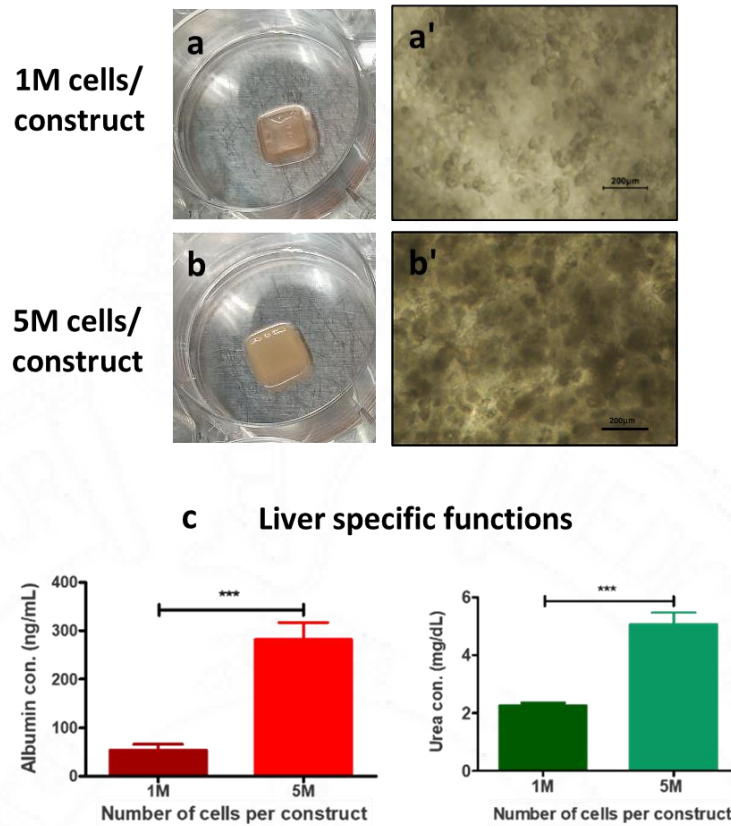


Figure 35. 3D Bioprinted liver constructs with GelMA-G-AO bioink. Bioprinted construct with (a & a') 1 Million (1M) hepatocytes/construct and (b & b') 5 Million (5M) hepatocytes/construct. Phase contrast images showing the cell density within the 1M and 5M cells/bioprinted construct, (c) Albumin and Urea secretion by bioprinted constructs on day 1. [Reproduced from Anupama Sekar *J et al* 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

Histological analysis by H&E staining of 3D bioprinted liver constructs with 1M and 5M cells/construct showed the arrangement of cells within the hydrogel. The cells in the 1M constructs were scattered and spread out with minimal cell-cell contact (Figure 36 a and a'). The 5M constructs showed better cell packing and cells with increased interactions between cells and a lower hydrogel content (Figure 36 b and b'). As mentioned earlier, normal liver constitutes a small portion of ECM with higher density of cells (Gordillo *et al.*,

2015). Therefore, 5M construct more closely resembled native liver tissue due to its higher cell density and lesser amount of hydrogel mimicking the lower ECM content with more cell-cell interactions.

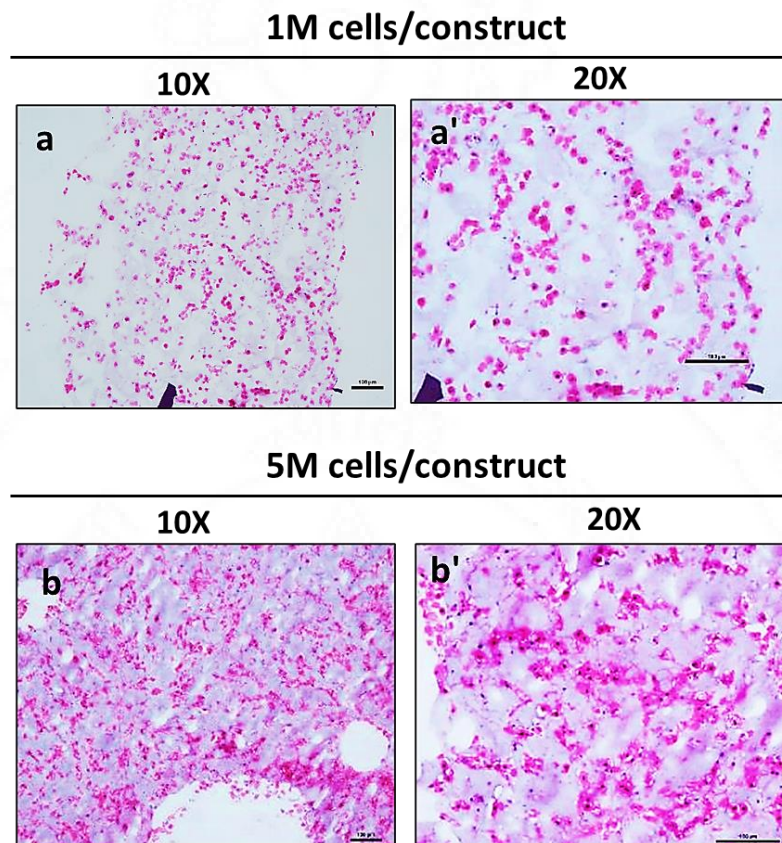


Figure 36. Histological analysis of bioprinted construct. H & E stained bioprinted constructs with 1M hepatocytes/construct (a & a') and bioprinted constructs with 5M hepatocytes/construct (b & b'). [Reproduced from Anupama Sekar J *et al* 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

Dvir-Ginzberg *et al.* reported a three-dimensional hepatocyte construct developed on alginate matrix (Dvir-Ginzberg *et al.*, 2003). They studied the significance of cell density on hepatocellular functions and demonstrated that relative to low cell density constructs, cellular constructs with a higher cell density displayed elevated liver-specific functions. Their study also showed that a higher

cell density of 5 million cells/cm³ maintained higher cell viability than those with low cell density. Therefore, findings of this study exhibiting, 5 million cells per construct (250 μ L) with dimensions of 10×10×2 mm is equivalent to 40 million cells/cm³. Therefore, based on the functional and histological evaluation, it can be deduced that 3D bioprinted constructs with 5M cells/construct showed enhanced performance than those with 1M cells/construct.

4.15.3 Functional evaluation of Bioprinted liver with high cell density

It is necessary to ensure functionality of hepatocytes within the bioprinted liver tissue for a longer duration *in vitro*. For *in vivo* evaluation of bioprinted liver construct, the bioprinted liver will be transplanted within 5 days post printing. Hence, the functional performance of the high cell density (5M) 3D bioprinted constructs in exhibiting liver-specific characteristics over an extended period of 5 days was studied. The result showed a linear increase in both albumin and urea over 5 days in culture (Figure 37a & b).

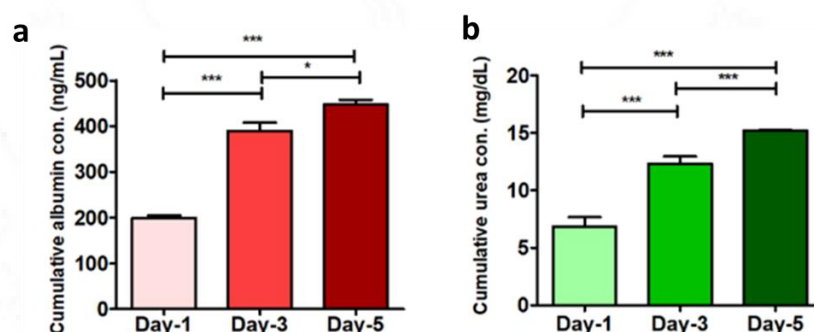


Figure 37. Liver specific functions expressed by high cell density bioprinted constructs with 5M cells/construct (a) albumin and (b) urea in 5M cells/construct over 5 days in culture. [Reproduced from Anupama Sekar J *et al* 2023 Biomed. Mater. 18 044101 DOI: 10.1088/1748-605X/acd82f]

4.16 Bioprinting of liver construct with modified bioink formulation

In vivo transplantation of hepatic spheroids encapsulated in hydrogels showed improvement in engraftment and survival of the cells (Lee *et al.*, 2021c, Abu-Absi *et al.*, 2002). There were also reports demonstrating the combined culturing of primary human hepatocyte (PHH) spheroids on hydrogel matrix providing optimal environment for the PHH spheroids, allowing them to maintain their differentiation and proliferative ability over a prolonged period of time (Rose *et al.*, 2021). High cell density culture improves the cell-cell contact by reduced distance between cells that favors formation of cell aggregates through matrix assisted spheroid formation (Zhang *et al.*, 2020, Cui *et al.*, 2017). The structure of these hepatic spheroids closely resembles the native liver tissue (Shibuya *et al.*, 2021).

The 5M cells/construct bioprinted in this study crosslinked using Irgacure 2959 as photoinitiator did not organize into spheroids was not observed, despite the high cell density and cell-cell interaction. In a study conducted by Villata, *et al.* it was reported that spheroid formation was observed in 7.5% and 10% GelMA formulations whereas not in 5% GelMA due to its low crosslinking degree (Villata *et al.*, 2023). This indicates that the formation of spheroids is highly influenced by the intrinsic properties of the hydrogel like crosslinking density thereby stiffness and pore size. Modifying the hydrogel components allows adjustment of microenvironment for cellular organization (Zhang *et al.*, 2020). It was also shown that hydrogel stiffness has an impact on spheroid formation i.e. too rigid matrix makes spheroid expansion challenging by hindering cell movement and

aggregation whereas soft and medium matrix encourages cell aggregation and spheroid formation. Therefore, without increasing the polymer concentration, the crosslinking density of hydrogel can be fine-tuned by either increasing the concentration of Irgacure (photoinitiator used) or by increasing UV exposure. However, higher concentration of Irgacure or increasing UV time can affect cell viability (Zhao *et al.*, 2020).

An alternative photoinitiator that is more water soluble than Irgacure 2959 used in GelMA crosslinking is Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (Nguyen *et al.*, 2019). Research has shown that hydrogels crosslinked in presence of LAP exhibit improved mechanical properties than hydrogels crosslinked in the presence of Irgacure due to the increased number of crosslinks between polymer chains (Elkhoury *et al.*, 2023). However, it has also been reported that LAP at concentration <0.5% was not found to be cytotoxic (Nguyen *et al.*, 2019). Therefore, for optimizing hydrogel for cellular organization, bioink formulation with 5% GelMA, 3% gelatin and antioxidants were mixed with 0.25% LAP as photoinitiator and was further analyzed.

4.16.1 ROS Activity

When the Irgacure was replaced with LAP as photoinitiator, the level of iROS produced during photo-crosslinking was measured to analyze the photoprotective effect of antioxidants on GelMA. The results showed that, in comparison to the base GelMA-G+LAP bioink formulation, the cells encapsulated in GelMA-G-AO+LAP exhibited only ROS activity of $45.59 \pm 2\%$ (Figure 38). Furthermore, it was also observed that the decline in ROS generation in GelMA-

G-AO+LAP followed a similar trend to that of reduction in ROS activity observed in GelMA-G-AO+Irg when compared with GelMA-G+Irg. Thus, regardless of the photoinitiators used, the results demonstrate that the addition of antioxidants in the GelMA bioink formulation offers photoprotection by reducing ROS generated during UV irradiation.

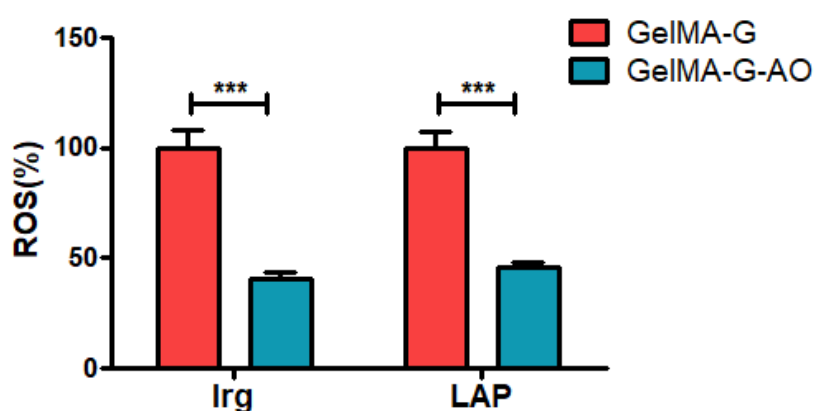


Figure 38. Intracellular ROS production in GelMA and GelMA-G-AO. Graph depicts the formation ROS under UV exposure in presence of Irgacure and LAP as photoinitiators ((*** $p < 0.001$)).

4.16.2 UV curing analysis

Dynamic crosslinking analysis shown in Figure 39 revealed that GelMA-G-AO+LAP showed a sudden increase in the storage modulus within 60 s of UV exposure indicating initiation of crosslinking and reached higher storage modulus within 100 s and maintained constant storage modulus till 200 s of UV exposure. Whereas in case of GelMA-G-AO+Irg, storage modulus was slowly increasing as UV exposure time increases and took 200 s to attain the storage modulus same as that of GelMA-G-AO+LAP attained within 100 s. The result was consistent with

a previous report that compared between Irgacure 2959 and LAP as photoinitiators for polyethyleneglycol diacrylate hydrogel and showed that LAP resulted in gelation times that were lower than Irgacure 2959 when exposed to 365 nm light at comparable concentrations and UV-A exposures (Fairbanks *et al.*, 2009).

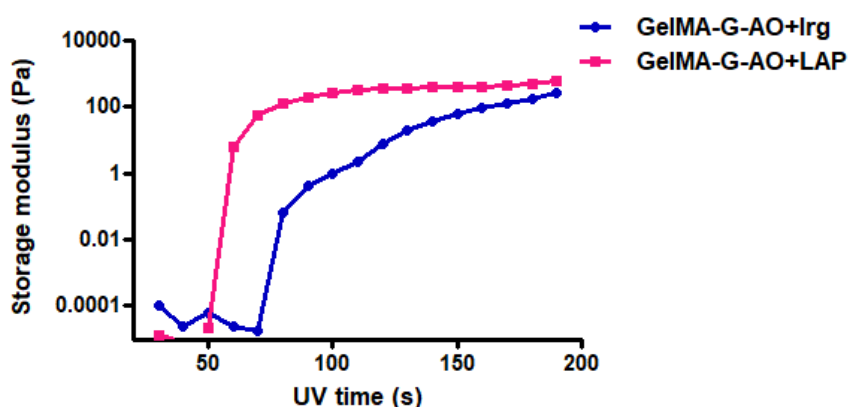


Figure 39. UV curing analysis of GelMA-G-AO. The graph shows the variation of storage modulus of GelMA-G-AO in presence of Irgacure and LAP as photoinitiators with respect to increasing UV exposure time from 50-200 s.

This is because in case of Irgacure 2959, the peak absorbance is at 280 nm with a tail absorption at 365 nm. This low absorbance at 365 nm makes photoinitiated polymerization kinetics of Irgacure 2959 inefficient whereas LAP has a maximum absorbance at 365nm and tail absorption in the visible range of 400–420 nm. Thus, LAP has improved initiation efficiency and the shorter light exposure times can yield sufficiently crosslinked hydrogels (Nguyen *et al.*, 2019).

4.16.3 Mechanical property

The crosslinked GelMA-G-AO+Irg and GelMA-G-AO+LAP hydrogel samples prepared for compression testing is shown in Figure 40. The compressive modulus of GelMA-G-AO+Irg and GelMA-G-AO+LAP was 3.9 ± 0.2 and $5.7 \pm$

0.4 kPa kPa respectively. Therefore, the findings indicate that the maximum absorption of LAP at 365 nm led to an increase in crosslinking density of GelMA-G-AO resulting in an enhancement in its mechanical strength. These results align with previous research that suggests hydrogels crosslinked with LAP demonstrate improved mechanical properties compared to those crosslinked with Irgacure, likely due to the increased number of crosslinks between polymer chains (Elkhoury *et al.*, 2023).

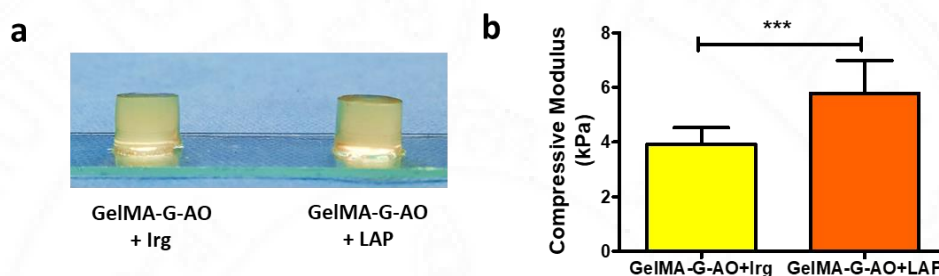


Figure 40. Analysis of mechanical property. (a) Photocrosslinked GelMA-G-AO+LAP and GelMA-G-AO+Irg hydrogels prepared for compression testing, (b) Graph showing a significant difference in compressive modulus between GelMA-G-AO+LAP and GelMA-G-AO+Irg. (***) represents p -value >0.001 .

4.16.4 Biological evaluation of GelMA-G-AO+LAP

It is necessary to evaluate the GelMA-G-AO formulation with 0.25% LAP for its cytotoxicity. *In vitro* cytotoxicity analysis of the GelMA-G-AO+LAP hydrogel based on ISO 10993-5 by direct contact with L-929 fibroblast cells did not show cytotoxic reactivity under or around the sample whereas the positive control showed severe cytotoxic reactivity and negative control did not exhibit cytotoxic reactivity (Figure 41 a).

HepG2 cells encapsulated in the GelMA-G-AO+LAP hydrogel was found to be viable after 24 h of culture which confirms that GelMA-G-AO with 0.25% LAP is non-cytotoxic (Figure 41b).

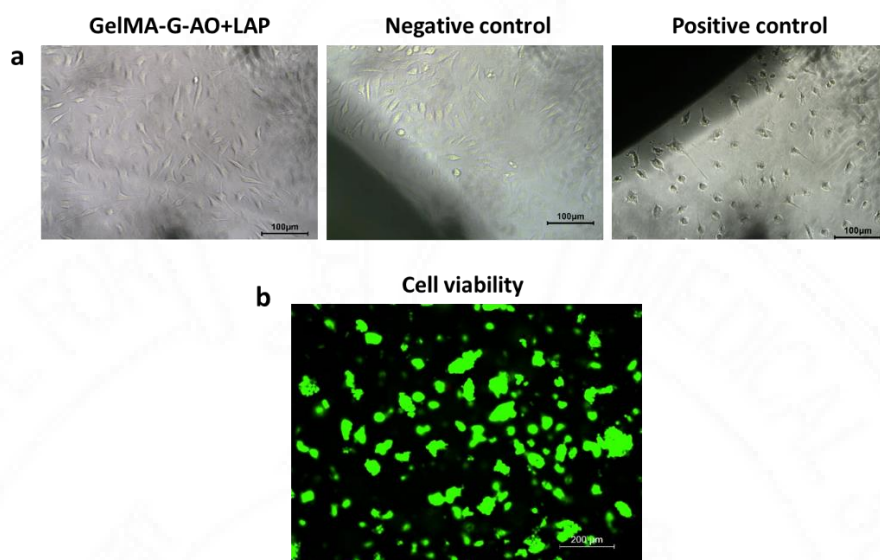


Figure 41. Biological evaluation of GelMA-G-AO+LAP (a) Direct contact test of GelMA-G-AO+LAP on L-929 cells (Scale bar 100µm), (b) Viability of HepG2 cells encapsulated in GelMA-G-AO+LAP (Scale bar 200µm).

4.16.5 Hepatic spheroid formation analysis

The encapsulation study showed that in GelMA-G-AO+LAP hydrogel, hepatocytes aggregate within 24 h of culturing to form spheroids of size $99 \pm 8 \mu\text{m}$ (Figure 42 a), and further increase in spheroid size ($159 \pm 11 \mu\text{m}$) and spheroid fusion was observed after 48 h (Figure 42 b). In contrast, the cells encapsulated in GelMA-G-AO+Irg despite showing cell-to-cell contact and distribution, cells did not aggregate into spheroid even after 48h of culture.

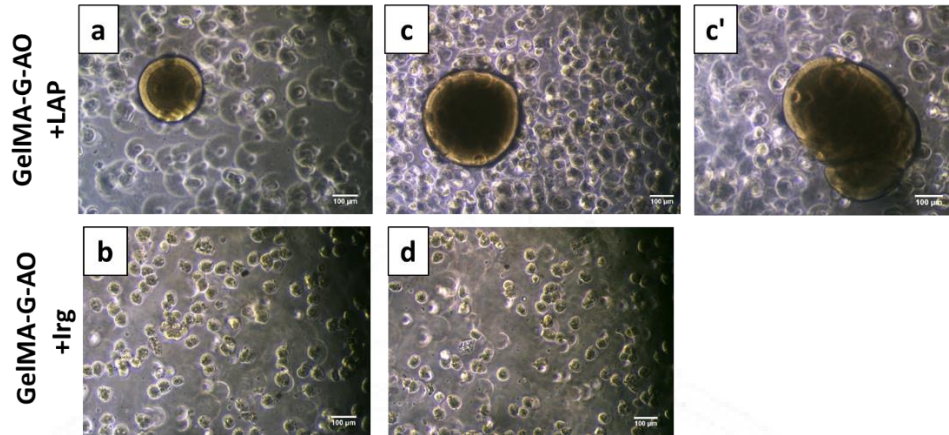


Figure 42. Spheroid formation analysis by cell encapsulation study. (a & b) Phase contrast image of the cells taken after 24 h and (c, c' & d) taken after 48 h when encapsulated in GelMA-G-AO+LAP and GelMA-G-AO+Irg respectively.

Cell aggregation is modulated by cell–cell contact rather than cell–matrix contact, and an overly strong cell–matrix adhesion would prevent cell aggregation. As previously discussed, there is correlation between hydrogel properties and spheroid formation. Therefore, the increase in crosslinking density of GelMA-G-AO when crosslinked with LAP has modified the hydrogel matrix to enable self-organization of cells into spheroids.

The size of the spheroids is also an important factor to be considered as spheroids require more oxygen and nutrients compared to single hepatocytes. Studies have shown that hepatic spheroids larger than 200 µm experiences a critical decrease in oxygen concentration at the core (Curcio *et al.*, 2007). Here, in GelMA-G-AO+LAP, hepatocytes formed spheroids with acceptable size of less than 200 µm.

4.16.6 3D Bioprinting with GelMA-G-AO+LAP formulation

It has previously been reported that bioprinting primary human hepatocytes and non-parenchymal cells on transwell culture inserts of multi-well plates resulted in spheroids with improved hepatic characteristics (Deborah G. Nguyen^{1*}, 2016). Therefore, 5×10^6 cells containing hepatocytes and NPC at a 4:1 ratio was formulated with GelMA-G-AO+LAP to 3D bioprint the liver construct (Figure 43 a).

The constructs immediately observed after bioprinting appeared as distributed single cells under phase contrast microscope (Figure 43b). After 24 h of incubation, large number of spheroids were observed that has mean diameter of $145 \pm 20 \mu\text{m}$ (Figure 43c and c'). On further culture up to 48 h, the number of spheroids increased with mean diameter up to $171 \pm 20 \mu\text{m}$ (Figure 43d and d'). The cellular organization of bioprinted construct analyzed by histological method showed compact plate like arrangement in spheroids. Multiple spheroids spaced $64.7 \pm 13 \mu\text{m}$ apart partially mimics the native liver.

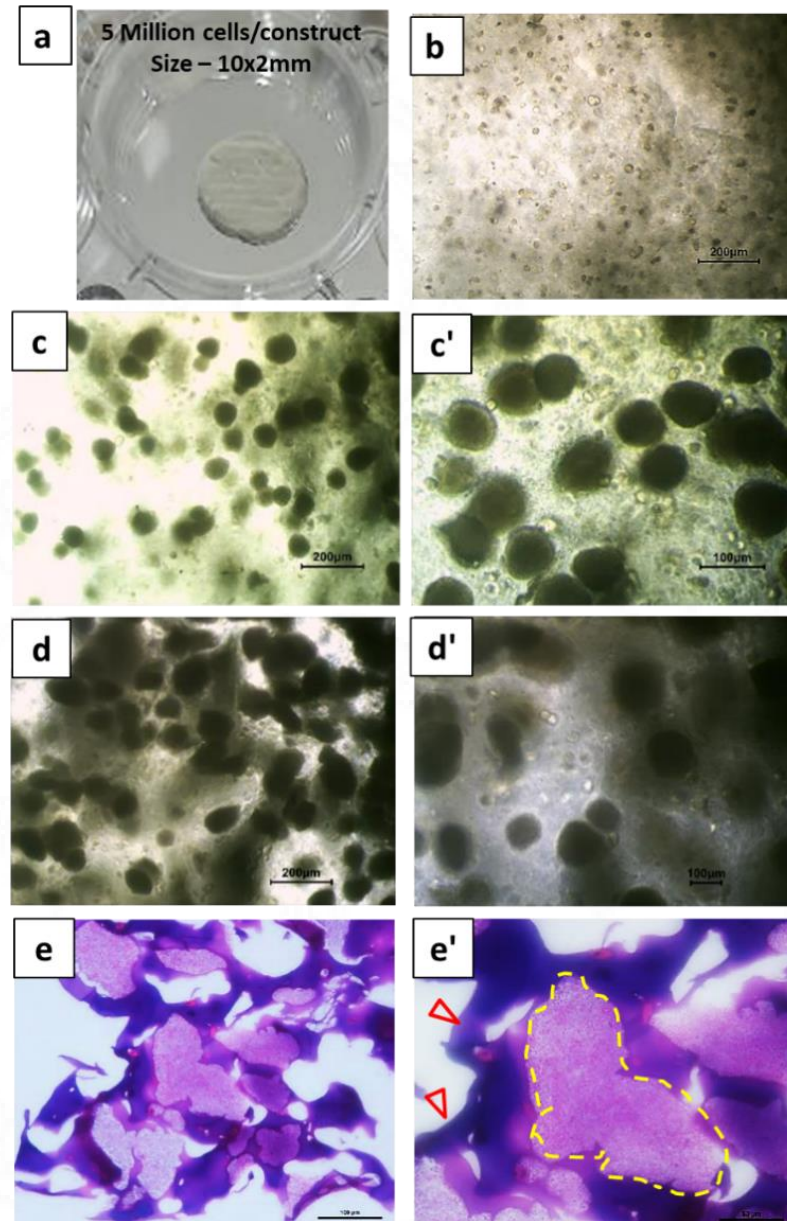


Figure 43. Spheroid formation in bioprinted liver construct. (a) Photograph of the bioprinted liver construct taken immediately after crosslinking. Phase contrast image of bioprinted construct at (b) 0h captured using 10X objective, (c & c') 24h captured using 10X and 20X objective, (d & d') 48h captured using 10X and 20X objective and (e & e') H and E-stained image of the bioprinted construct cultured for 48h captured using 10X and 20X objective. Red arrow head indicates the hydrogel and yellow dotted line represents the spheroid within the bioprinted construct.

In comparison to cells encapsulated in hydrogel shown in Figure 42a, it was noted that a higher number of spheroids were formed in the bioprinted

construct. In hydrogel structures, oxygen and nutrient supply to cells relies on diffusion through the culture medium. It has been reported that the larger spheroids dimension in grid architecture could be attributed to the better exchange of oxygen and nutrients, due to the higher surface area exposed to the cell culture medium (Villata *et al.*, 2023). Similarly, in another study it was also shown that the cells in the inner region of a consolidated hydrogel layer in the sandwich model experience impaired medium supply. As a result, the interconnected microchannels in the 3D Printed grid structure allow for a greater inflow of culture medium than the sandwich model, which facilitates mass transfer and more cell growth (He *et al.*, 2022).

4.17 Biocompatibility evaluation of 3D printed construct

The *in vivo* biocompatibility of 3D Printed GelMA-G and GelMA-G-AO construct was evaluated by ISO 10993-6 Biological Evaluation of Medical Devices”- Tests for local effects after implantation. The steps in the surgical procedure of subcutaneous implantation in rat is shown in Figure 44b.

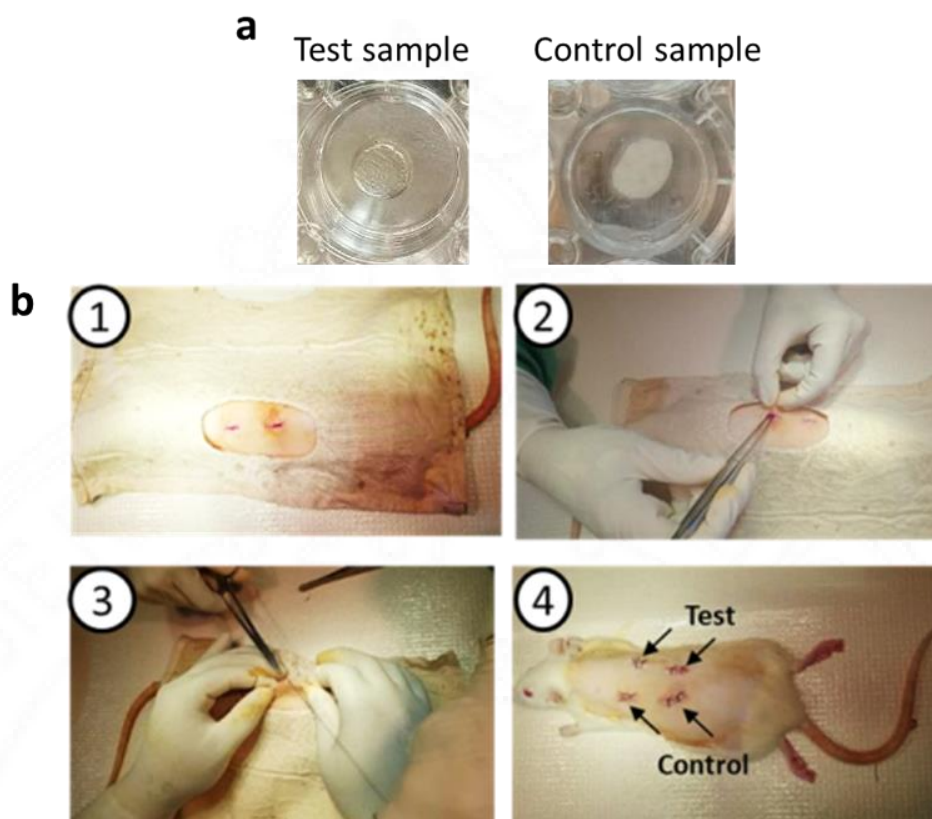


Figure 44. Subcutaneous implantation of 3D printed construct. (a) 3D printed disc shaped construct of dimension 10×2 mm and collagen sample soaked in PBS prepared for implantation. (b) Steps in subcutaneous implantation surgery 1) Incision on dorsal skin surface, 2) Sample insertion on subcutaneous pocket, 3) Suturing of incision and 4) Implanted sites

Subcutaneous implantation of 3D printed GelMA-G+LAP and GelMA-G-AO+LAP construct on rat models did not show any signs of mortality or morbidity in animals. The surgical wound in the implantation site was completely healed (Figure 45a). There were no gross pathological observations during necropsy in any tissue.

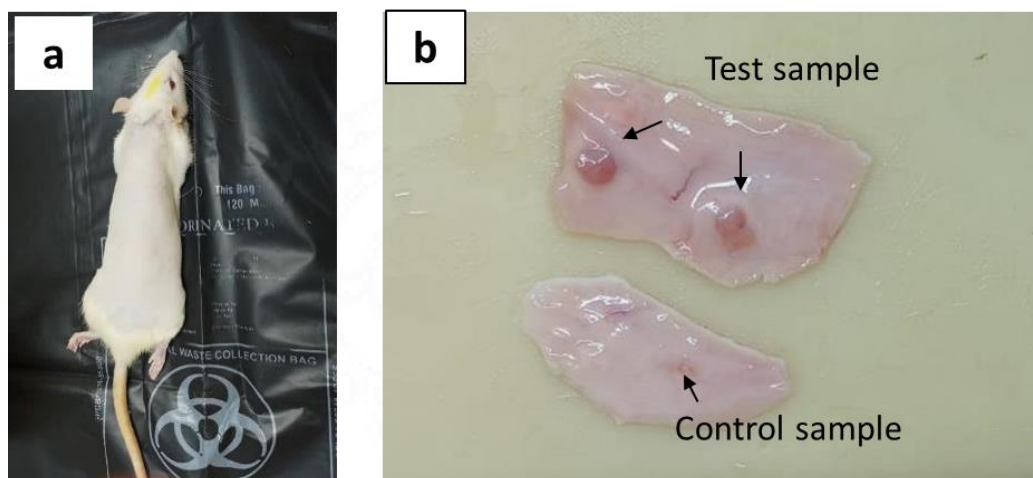


Figure 45. Observation at the end of experiment (a) Photograph of the rat captured on day 28 indicating complete wound healing post-surgery (b) Gross pathology of the implanted subcutaneous tissues.

Gross observation of implantation site after 28 days showed no signs of inflammation, edema or toxicity in the tissue surrounding the implants. All test samples were easily identifiable at the implanted site (Figure 45b). On the other hand, control samples (Collagen) were not clearly visible at the site, indicating that complete biodegradation that made collagen undistinguishable at the implanted area.

In accordance with the gross observation, histological analysis of the collagen implanted site also revealed that there were no traces of material in the implantation site as well as no signs of foreign body reaction in the implanted site or surrounding tissues (Figure 46).

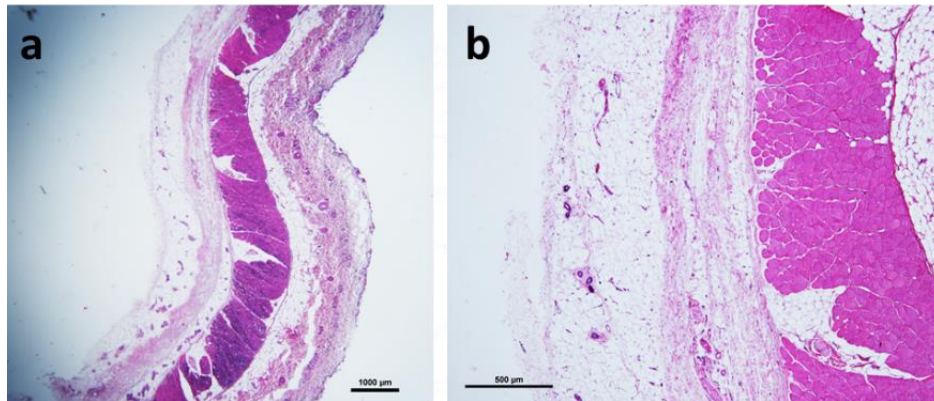


Figure 46. Histological examination of control implanted sites by H & E staining. (a) Images captured using 10X objective (scalebar 1000µm) and (b) captured using 20X objective (scalebar 500µm)

In the case of both test samples (GelMA-G+LAP and GelMA-G-AO+LAP) implanted site, histological analysis indicated the presence of residual hydrogel in the tissue (Figure 47b and f) which confirms that the 3D printed construct has not completely degraded upon implantation for 28 days. The presence of fibrous capsule was also observed in the implantation site of GelMA-G+LAP and GelMA-G-AO+LAP constructs (Figure 47b and f).

Histological analysis also revealed the formation of neovascularization in the implanted tissue site of both GelMA-G+LAP and GelMA-G-AO+LAP (Figure 47c and g). Foreign body giant cells (FBGC) surrounding the material, and the presence of other immune cells were also seen in the test sample implanted sites (Figure 47d and h).

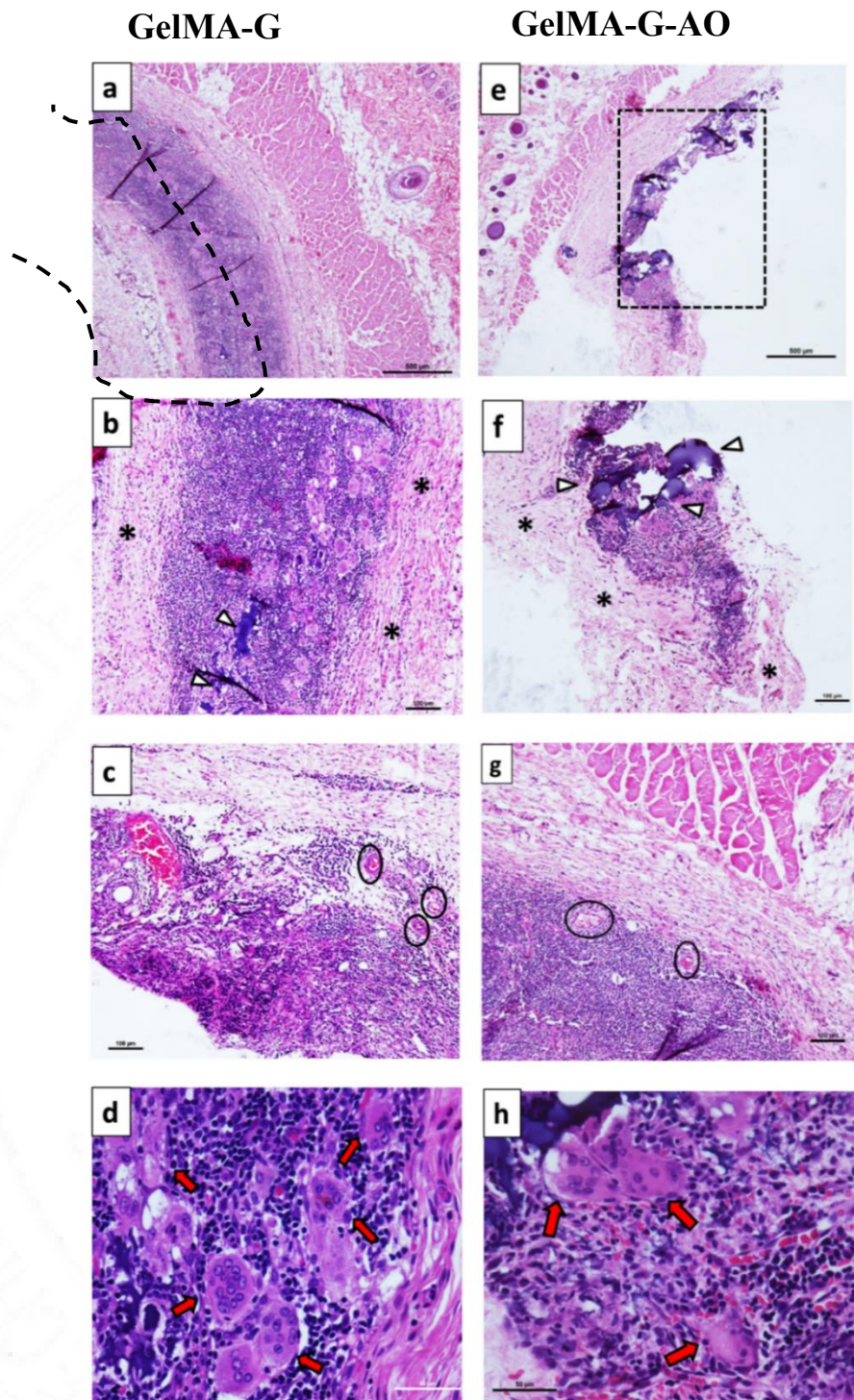


Figure 47. Histological examination of 3D printed construct implanted sites. (a & e) Dotted line depicts GelMA-G and GelMA-G-AO implanted sites respectively, (b & f) Asterisks indicates fibrous capsule and arrow head marks the hydrogel residues, (c & g) black circle shows the presence of neovascularization and (d & h) red arrows indicate FBGC in GelMA and GelMA-G-AO implanted sites respectively.

When a biomaterial is implanted into a body, foreign body reaction (FBR) is initiated as a result of host response and the understanding of the FBR is important to determine the efficiency of tissue-engineered construct for *in vivo* application. The events in the foreign body reaction include injury, blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granular tissue development and the end stage of the FBR and healing response is fibrous encapsulation (Anderson *et al.*, 2008). The nature, structure, size and form of the implanted biomaterials, the tissue area of implantation, and period of observation after implantation influences the tissue response to biomaterials (Lužajić Božinovski *et al.*, 2022). In case of biodegradable materials, they are eventually metabolized by the body and eliminated through physiological processes, hence they do not show persisting FBR or do not cause fibrous capsule formation (Li and Zreiqat, 2019). Therefore, the absence of FBR at the control site may be due to the complete biodegradation of collagen, while the FBR and fibrous capsule at the test site is likely due to ongoing biodegradation of GelMA hydrogel within 28 days of implantation.

The formation of fibrous capsule around the implant may confine the implant and prevents it from interacting with the surrounding tissue. In order to ensure cell survival of cell-laden hydrogel *in vivo*, adequate vascularization is required. Therefore, the thickness and vascularity of the capsule influence the overall mass-transfer resistance of the tissue. Thicker or more dense capsules with minimal vascular density may hinder the diffusion of analytes to and from the implanted tissue construct. Tissues within the fibrous capsule develop a larger and more complex network of blood vessels to guarantee proper oxygen and nutrient

supply (Bartoli *et al.*, 2010). Inflammatory cells may serve as a contributing factor in the formation of angiogenic and neovascular responses *via* the production of various angiogenic substrates. Here within fibrous capsule around implanted hydrogel, presence of neovascularization seems to be promising for biodegradation *in vivo*.

One of the immune cells formed during FBR are macrophages that acts as a phagocyte and engulfs small particles of foreign substances and when the size of material becomes larger ($>100\mu\text{m}$), macrophages cannot engulf them, so they fuse together and form foreign body giant cells. Also, it has been reported that macrophages and FBGCs may persist for the lifetime of the implant and releases mediators for biomaterial degradation (Sheikh *et al.*, 2015). Therefore, the presence of FBGC further confirms that GelMA-G and GelMA-G-AO hydrogel was not completely degraded at time point of 28 days. It has been reported that as the degree of methacrylation increases, the biodegradation time of GelMA structures also increases (Koshy *et al.*, 2014) (Nguyen *et al.*, 2015). Thus, the histological examination suggests that both GelMA-G and GelMA-G-AO elicited similar immune response when implanted subcutaneously in rat.

4.18 In vivo evaluation of 3D bioprinted liver construct

For evaluating the efficacy of bioprinted liver construct with GelMA-G-AO+LAP, bioprinted rat liver (5M cells/construct) of cylindrical shape and size $10 \times 2 \text{ mm}$ was transplanted into rat liver. Figure 48 shows the surgical steps involved in the transplantation of bioprinted liver.

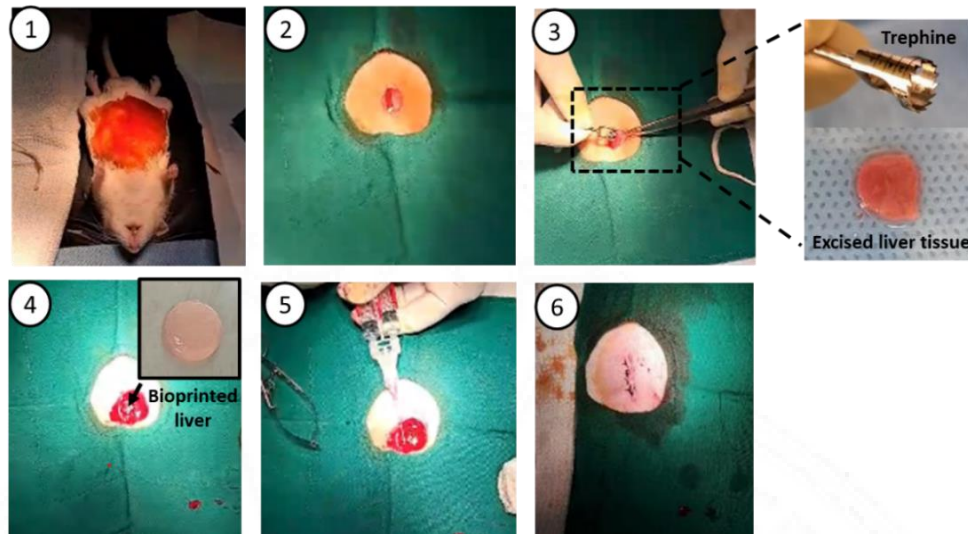


Figure 48. Surgical steps in the transplantation of 3D Bioprinted Liver into rat liver. 1) Anaesthetized rat, 2) Laparotomy 3) Creation of defect site 4) Transplantation of bioprinted liver 5) Application of fibrin glue and 6) Sutured incision

All the six animals recovered from the bioprinted liver transplantation surgery on the following day, and the stitches were fully healed by day 7. The post-operative observation up to 28 days showed that all the transplanted animals were healthy without any sign of tissue rejection. The body weights of the animals were as normal as they were consuming required food and water.

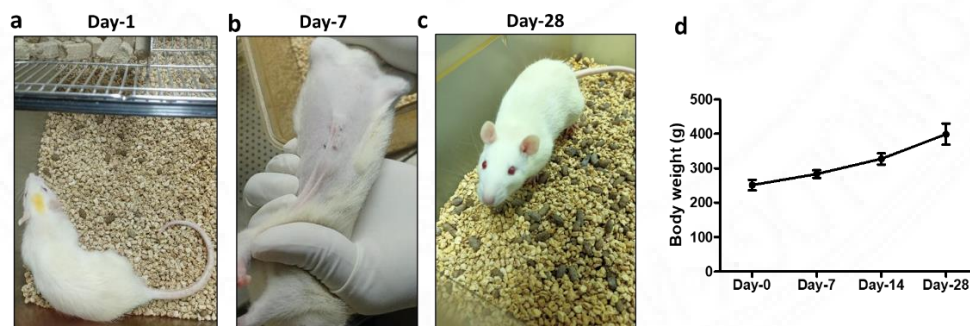


Figure 49. Post-transplantation observations. (a) Liver construct transplanted animal on day 1 post-surgery, (b) completely healed suture observed on day 7, (c) animal on day 28 (d) Graph showing the increase in body weight of animals from day 0 to 28.

4.18.1 Biochemical analysis

Liver function tests (LFTs) indicate the health of the liver from the quantitative information of proteins and enzymes in the blood. Any change in these biochemical factors from normal range can indicate problems of liver. Here, liver specific biochemical parameters were analyzed from serum samples collected before and after transplantation in order to assess whether bioprinted liver transplantation surgery and intake of immunosuppressants has affected the normal functioning of the host liver. The reduction of serum albumin from the normal range is a significant characteristic of liver damage (Lee *et al.*, 2017). According to the LFT on serum, the secretion of proteins, albumin and urea (Figure 50a, b and c) did not exhibit a significant difference on days 7, 14 and 28 when compared with day prior to surgery (day 0).

Post operative changes in liver enzymes may indicate loss of liver function or graft rejection. Aspartic aminotransferase (AST also known as glutamic oxaloacetic transaminase [GOT]), Alanine aminotransferase (ALT also known as glutamate pyruvate transaminase [GPT]) and Alkaline phosphatase (ALP) are normally found in low levels in the blood. Therefore, elevated levels of AST, ALT and ALP are considered as sensitive indicators of liver damage (Lee *et al.*, 2017). The results shown in Figure 50d indicates that the level of Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT) and ALP observed on days post-surgery remained within the normal range as seen in the animals prior to surgery. As a result, the liver function test shows proper functioning of liver with no signs of graft rejection.

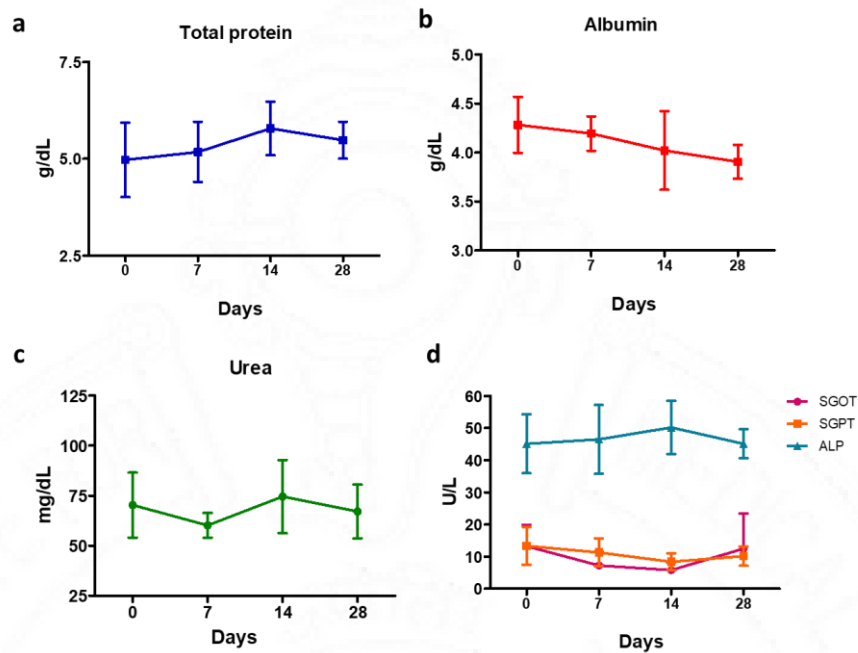


Figure 50. Serum analysis. Graphs depicts the non-significant difference in liver functions from day 0 to 28.

4.18.2 Gross pathology

Animals were euthanized after 28 days post transplantation by CO₂ asphyxiation and the implant site was surgically retrieved. There were no gross pathological observations during necropsy in any of the tissue including the transplantation site and the transplant was intact at the implant site (Figure 51).



Figure 51. Gross photographs of the liver with the transplant

4.18.3 Histology

One of the main barriers to the clinical application of tissue-engineered implants is inadequate vascularization. Lack of vascularization due to poor graft integration can result in insufficient oxygen and nutrient supply, leading to necrosis around implant and failure (Masson - Meyers *et al.*, 2021). It is also known that the implantation of any foreign material into the body causes cellular and tissue responses. A series of events, such as interaction with blood components, protein layer deposition on the biomaterial, formation of a provisional matrix, immune cell recruitment, acute and chronic inflammation, granulation tissue formation, foreign body reaction, fibrosis, and fibrous capsule development, are triggered when an implanted material interacts with host tissue (Kämmerling *et al.*, 2021). Therefore, tissue regeneration and healing after biomaterial implantation depend on the host immune response; however, an excessive response from the host can eventually lead to biomaterial failure.

The histological analysis by H&E staining of transplanted liver tissues with 3D bioprinted liver construct showed efficient integration of transplanted bioprinted liver into the host liver (Figure 52a and b). The blood vessels were observed extending from the host tissue into the transplanted bioprinted construct (Figure 52c). The tissue repair was evidenced by the presence of microvasculature within the bioprinted liver (Figure 52d).

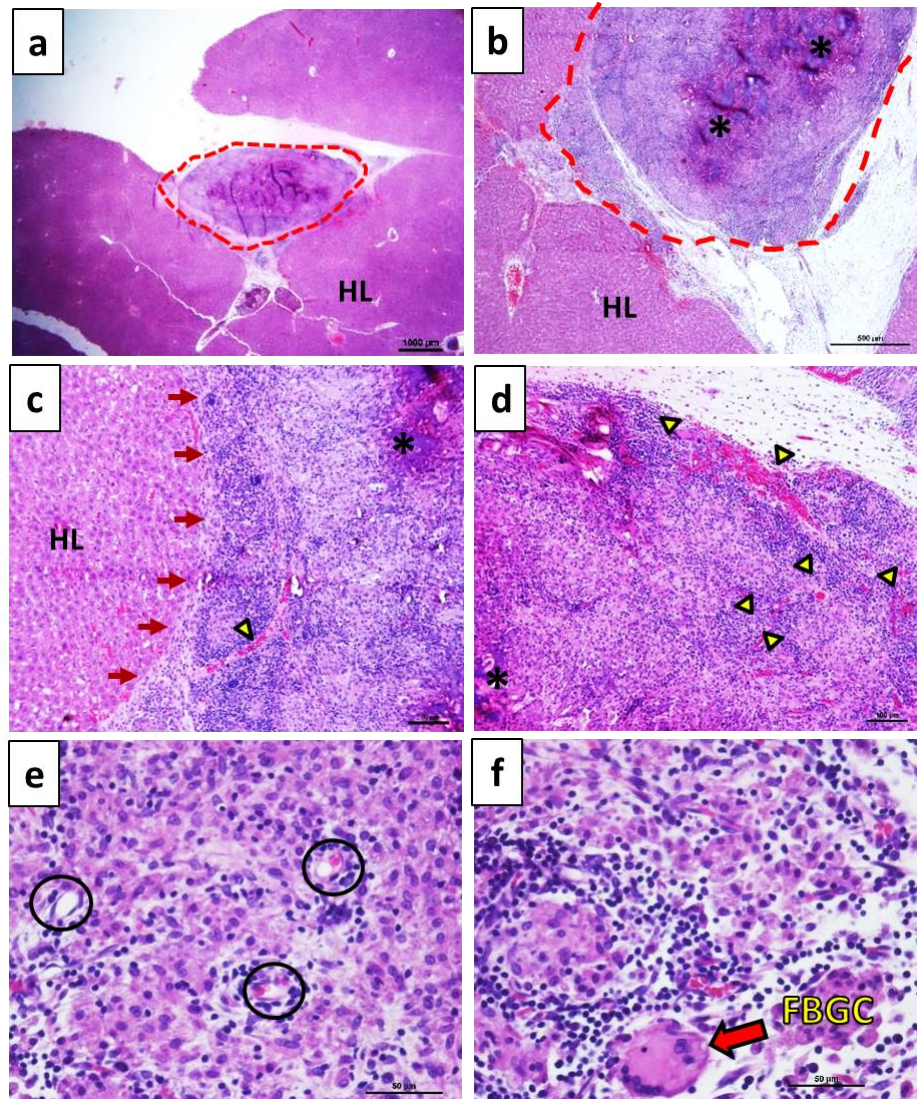


Figure 52. Histological details showing integration of 3D Bioprinted liver in rat liver. (a & b) Images showing transplantation site, (c) Arrows indicating host tissue-implant interface and arrow head shows vascularization into the transplant (d) Arrow head indicates vascularization inside the bioprinted construct, (e) Neovascularization sites indicated by black circles and (f) Arrow indicating foreign body giant cells.

Histological analysis also revealed the presence of neovascularization in the transplanted site (Figure 52e). Tissue exposure to biomaterials results in foreign body response and the formation of foreign body giant cells (FBGCs) in

the transplanted site indicating immune cell infiltration was also confirmed by histo-analysis (Figure 52f).

The process of newly formed blood vessels growing into an implanted tissue construct is called vascularization, and it is a very dynamic process. The first stage of this process involves the expression of pro-angiogenic factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) etc. that are stimulated by the wound environment activates the host microvasculature at the implantation site. The activation of angiogenesis initiates endothelial cells in the host microvasculature to produce matrix metalloproteinases and degrades basement membrane causing the host microvasculature to move into the surrounding interstitium which is displayed through the development of vascular buds and sprouts. Thus, as time progresses, the sprouts develop into the implanted tissue construct and establish connections with one another to create new blood-perfused microvascular networks (Laschke and Menger, 2012).

The presence of immune cells would be necessary for successful vascularization of a transplanted tissue construct to promote angiogenesis and nourish the transplanted cells. Studies have shown that macrophages enhances wound healing through the release of enzymes important for tissue reorganization and angiogenesis (Salthouse *et al.*, 2023). Therefore, the angiogenic growth factors can be produced by the host tissue as a result of an inflammatory response to the implant or as a result of tissue damage occurred during the transplantation process (Laschke and Menger, 2012).

It is also important to note that GelMA, the hydrogel component in the bioprinted construct retains the RGD motifs of gelatin despite the modification with methacrylation and presence of RGD motifs initiates adherence of endothelial cells which aids in vascularization (Heltmann-Meyer *et al.*, 2021). Thus, the histological examination reveals that along with the immune cells, the presence of RGD motifs in GelMA hydrogel might have also favored vascularization in the transplanted bioprinted construct.

In the case of large sized implanted biomaterials, macrophages cannot engulf them, so macrophages fuse together and form a large sized cells known as foreign body giant cells (Sheikh *et al.*, 2015). The histological examination of transplanted liver showed the presence of macrophages and FBGC without the presence of fibrous capsule around the transplant site (Figure 52f). It has been reported that macrophages and FBGCs may persist for the lifetime of the implanted biomaterial and releases mediators for biomaterial degradation (Sheikh *et al.*, 2015).

Therefore, the histological studies suggests that there was no adverse immune reaction to the allogenic cell laden constructs and efficient vascularization was achieved in the transplanted bioprinted liver when examined after 28 days of transplantation.

Chapter 5

SUMMARY AND CONCLUSION

5.1 Summary

Three-dimensional liver bioprinting is an emerging technology in the field of regenerative medicine that aids in the creation of functional tissue constructs that can be used as transplantable organ substitutes. Gelatin Methacrylamide (GelMA) which forms covalently cross-linked hydrogels under UV light ($\lambda=365$ nm) exposure in the presence of a photoinitiator, is a widely used hydrogel in bioink formulations for 3D bioprinting. After 3D bioprinting, the cell-laden constructs were exposed to UV light and during this photo-crosslinking of GelMA, a significant number of free radicals are produced and it is well established that exposure to UV light has a physiological impact by forming reactive oxygen species inside cells. The present study puts forward for the development of GelMA bioink with photoprotective property for the biofabrication of liver tissue constructs.

Towards achieving this goal, various objectives were formulated and were successfully accomplished. The first and second objective was to synthesize, characterize and evaluate GelMA formulation with photoprotective property for its suitability as a bioink for 3D bioprinting of liver constructs. The phototoxicity analysis in cells encapsulated in GelMA showed significantly higher ROS levels

than cells not exposed to UV light. To overcome this drawback, GelMA bioink was modified by incorporating cocktail of antioxidants such as Ascorbic acid and α -Tocopherol in order to provide protection to the cells from ROS. Analysis of ROS production in cells encapsulated in GelMA loaded with a cocktail of Ascorbic acid and α -Tocopherol showed a significant influence in reducing intracellular ROS compared to either antioxidant used alone. Also, GelMA and its photoprotective bioink formulation exhibited good cytocompatibility, excellent printability and shape fidelity.

To achieve the highest level of functional performance in bioprinted constructs, it is necessary to mimic the cell arrangement and microenvironment of the native tissues. Hence, it would be preferable to consider the high cell density of hepatocytes and a low ECM compartment in biofabricated tissue. Therefore, bioprinted liver construct with 5 million cells/construct was biofabricated and it showed better cell-cell interaction and functionality. Additionally, a modification in the photoinitiator used from Irgacure to LAP, hydrogel matrix promoted self-organization of cells encapsulated in hydrogel into spheroids. After *in vitro* studies, the third objective was to evaluate biocompatibility of 3D Printed constructs *in vivo*.

The final objective of the study was to evaluate efficiency of bioprinted liver followed by transplantation into rat liver. The biochemical and histological studies suggests that there was no adverse immune reaction to the allogenic cell laden constructs and efficient vascularization was achieved in the transplanted bioprinted liver when examined after 28 days of transplantation.

The following points highlights the outcomes of the study

1. A novel photoprotective GelMA based bioink (GelMA-G-AO) was formulated for fabrication of bioprinted liver constructs with high cell density.
2. The modification of hydrogel by increasing the crosslinking density of GelMA-G-AO with LAP as photoinitiator has allowed the hydrogel matrix to self-organize the cells to form spheroids within the bioprinted construct.
3. The *in vivo* transplantation of the allogenic cell laden bioprinted liver constructs into rat liver showed efficient tissue integration into host liver and vascularization in the transplanted bioprinted liver when examined after 28 days of transplantation.

5.2 Conclusion

Depending upon the results and inferences of the study, it can be concluded that

1. The addition of antioxidants in GelMA bioink formulation could reduce ROS production during photo-crosslinking and thereby provides photoprotection to the cells.
2. The GelMA formulated with antioxidant cocktail system exhibited ideal bioink properties for bioprinting liver tissue constructs.

3. Bioprinting of liver tissues with high cell density exhibited improved liver specific functions and histological resemblance with native liver tissues.
4. *In vivo* transplantation study of bioprinted liver showed efficient tissue integration into the host liver thereby providing a promising alternative to donor organs for transplantation.

Chapter 6

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Annexure

List of publications

Research articles

1. **Anupama Sekar J**, Shiny Velayudhan, and Anil Kumar PR "Biocompatibility evaluation of antioxidant cocktail loaded gelatin methacrylamide as bioink for extrusion-based 3D bioprinting." Biomedical Materials 18.4 (2023): 044101.

Book chapters

1. **Anupama Sekar, J., et al.** "3D bioprinting in tissue engineering and regenerative medicine: current landscape and future prospects." Biomaterials in Tissue Engineering and Regenerative Medicine: From Basic Concepts to State of the Art Approaches (2021): 561-580.
2. Pai, Roopesh R., **J. Anupama Sekar**, Shilpa Ajit, Shiny Velayudhan, Naresh Kasoju, and Anil Kumar PR. "Three-dimensional bioprinting of tissues and organs." In Biomedical Product and Materials Evaluation, pp. 135-150. Woodhead Publishing, 2022.

Patent filed

Three Dimensional Bioprinted *in vitro* Hepatotoxicity Assay Platform,
Filed on 23-03-2023: Publication awaited

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Educational qualification	M.Sc (Biotechnology) Amrita University, Kollam, Kerala, India.	2014-2016
	B.Sc (Biotechnology) Amrita University, Kollam, Kerala, India.	2011-2014

Research area

My PhD research focused on the development and functional evaluation of a modified form of gelatin based bioink for 3D bioprinting of liver tissue constructs. The study focused on formulating bioink based on GelMA with photoprotective property to avoid the harmful effect of photocrosslinking process. The procedure for synthesis of GelMA was optimised. The bioink with photoprotective components were formulated and evaluated for physicochemical properties by rheology, printability analysis and biological evaluation by *in vitro* cytotoxicity, cytocompatibility studies and functionality. The study also explored the biocompatibility and efficacy evaluation of the in-house synthesized bioink by *in vivo* studies in rat model.

Academic projects completed

Title of the project	Institute	Course	Year	Duration
Production, purification and application of naringinase from <i>Aspergillus</i> sp. section <i>nigri</i>	Amrita School of Biotechnology	B.Sc.	2014	6 months
Signaling pathways regulating chemoresistance and self-renewal in oral cancer	Rajiv Gandhi Centre for Biotechnology	M.Sc	2016	6 months

- BSc project was done under the guidance of Mr.Ajith Madhavan , Asst.Professor of Amrita School of Biotechnology, Kollam

- MSc project was done under the guidance of Dr. Tessy Thomas Maliekal, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram.

Awards

Name of the Award – Year	Details
Merit award – 2024	Best paper publication from Sree Chitra Tirunal Institute for Medical Sciences and Technology
Joint CSIR UGC NET Exam-2017	Qualified National Eligibility Test (NET) for Junior Research Scholarship (JRF) and Eligibility for Lectureship in the join exam of Council of Scientific & Industrial Research (CSIR) and University Grants Commission (UGC) with 59 th rank under CSIR category
GATE -2017	Qualified GATE (Graduate Aptitude Test in Engineering) in Biotechnology

Conference presentations

- Oral Presentation - Printability evaluation of hydrogel for extrusion-based 3D Bioprinting in the ICBMI 2020 International Conference held on Dec 6-9th 2020.
- Poster Presentation - Biocompatibility evaluation of Antioxidant enriched bioink for 3D Bioprinting of Liver constructs in the New Horizons in Biotechnology (NHBT) International Conference held on Nov 26-29th 2023.

Publications

• Research articles

1. **Anupama Sekar J**, Shiny Velayudhan, Senthilkumar Muthusamy and Anil Kumar PR "Silymarin enriched gelatin methacrylamide bioink imparts hepatoprotectivity to 3D bioprinted liver construct against carbon tetrachloride induced toxicity." European Journal of Pharmaceutics and Biopharmaceutics (2024): 114272.
2. **Anupama Sekar J**, Shiny Velayudhan, and Anil Kumar PR "Biocompatibility evaluation of antioxidant cocktail loaded gelatin methacrylamide as bioink for extrusion-based 3D bioprinting." Biomedical Materials 18.4 (2023): 044101.
3. Asha V. Nath, Shilpa Ajit, **Anupama J. Sekar**, Anil Kumar PR, and Senthilkumar Muthusamy. "MicroRNA-200c/429 mediated regulation of Zeb1 augments N-Cadherin in mouse cardiac mesenchymal cells." Cell Biology International 46, no. 2 (2022): 222-233.
4. Roopesh R.Pai, Shilpa Ajit, **Anupama Sekar J**, Sarath S. Nair, Anil Kumar PR and Shiny Velayudhan. "Radical scavenging gelatin methacrylamide based bioink formulation for three dimensional bioprinting of parenchymal liver construct." Bioprinting 27 (2022): e00214.

- **Abstract**

Anupama Sekar, J. and Anil Kumar P.R., Hepatoprotective Bioink for 3D Bioprinting of Liver, *Tissue Engineering Part A* 2022; 28(S2 Abstracts); 1-408.

- **Book chapters**

1. **Anupama Sekar, J.**, et al. "3D bioprinting in tissue engineering and regenerative medicine: current landscape and future prospects." *Biomaterials in Tissue Engineering and Regenerative Medicine: From Basic Concepts to State of the Art Approaches* (2021): 561-580.
2. Pai, Roopesh R., **J. Anupama Sekar**, Shilpa Ajit, Shiny Velayudhan, Naresh Kasoju, and Anil Kumar PR. "Three-dimensional bioprinting of tissues and organs." In *Biomedical Product and Materials Evaluation*, pp. 135-150. Woodhead Publishing, 2022.
3. Muthusamy, S., Ajit, S., Nath, A.V., **Anupama Sekar, J.** and Ramya Lakshmi, T.S., 2022. Enzymes from genetically modified organisms and their current applications in food development and food chain. *Novel Food Grade Enzymes: Applications in Food Processing and Preservation Industries*, pp.357-382.

- **Patent filed**

Three Dimensional Bioprinted *in vitro* Hepatotoxicity Assay Platform,
Filed on 23-03-2023: Publication awaited

Biomedical Materials



PAPER

Biocompatibility evaluation of antioxidant cocktail loaded gelatin methacrylamide as bioink for extrusion-based 3D bioprinting

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Keywords: 3D bioprinting, antioxidants, bioink, GelMA, hepatocytes

Supplementary material for this article is available [online](#)

Abstract

Three-dimensional (3D) liver bioprinting is a promising technique for creating 3D liver models that can be used for *in vitro* drug testing, hepatotoxicity studies, and transplantation. The functional performance of 3D bioprinted liver constructs are limited by the lack of cell–cell interactions, which calls for the creation of bioprinted tissue constructs with high cell densities. This study reports the fabrication of 3D bioprinted liver constructs using a novel photocrosslinkable gelatin methacrylamide (GelMA)-based bioink formulation. However, the formation of excess free radicals during photoinitiation poses a challenge, particularly during photocrosslinking of large constructs with high cell densities. Hence, we designed a bioink formulation comprising the base polymer GelMA loaded with an antioxidant cocktail containing vitamin C (L-ascorbic acid (AA)) and vitamin E (α -tocopherol (α -Toc)). We confirmed that the combination of antioxidants loaded in GelMA enhanced the ability to scavenge intracellular reactive oxygen species formed during photocrosslinking. The GelMA formulation was evaluated for biocompatibility *in vitro* and *in vivo*. These results demonstrated that the bioink had adequate rheological characteristics and was biocompatible. Furthermore, when compared to bioprinted constructs with lower cell density, high-density primary rat hepatocyte constructs demonstrated improved cell-cell interactions and liver-specific functions like albumin and urea secretion, which increased 5-fold and 2.5-fold, respectively.

1. Introduction

Liver is the second largest organ in human body that performs wide range of functions essential for normal body function. Therefore, a significant decline in liver function is detrimental to human health. Orthotropic liver transplantation is a tried and true treatment option for acute or end-stage liver diseases. However, scarcity of organ donors, high invasiveness and difficulties associated with life-long immune-suppressants has limited its benefits and increased the demand for engineering liver tissues [1].

To date various biofabrication strategies have been exploited to engineer functional liver that can

closely resemble their natural counterparts in terms of both architecture and functions. Three dimensional (3D) bioprinting is one such cutting-edge technology, which has the potential to precisely deposit biomaterials and cells in a layer-by-layer manner, to create heterogeneous tissue-mimetic constructs that promote cell–cell and cell-extracellular matrix (ECM) interactions in a 3D environment [2]. Liver is a complex organ, comprising of a variety of cell types at varying densities. Liver is organized into lobules and each lobule has well-arranged cellular architecture with majority of hepatocytes, that is arranged into hepatic chords separated by sinusoids [3]. Within the normal liver, the ECM comprises very limited compartment

which is less than 3% of the relative area on liver section [4]. Thus, liver has a high cellular density (1.3×10^8 cells per gram of tissue) and these densities need to be replicated during bio-printing to mimic the native tissue functionally and physiologically. The fabrication of high-density tissue constructs, on the other hand, is dependent on the bioprinting modality and bioink used.

Only very few methods for creating bioprinted tissues with high cell densities have been documented in the literature. Bedossa *et al* has reported the development a bioprinting approach to enable high-resolution transfer of spheroids into self-healing support hydrogels permits their patterning and fusing into high-cell density microtissues with predetermined spatial organization [5]. Daly *et al* on the other hand reported laser assisted bioprinting of engineered tissue with high cell density and successfully used this printing technology to print precisely miniaturized tissue like layouts with de novo high cell density and micro scale organization [6].

Extrusion bioprinters allow printing of tissue spheroids with 8000 cells in 300 μ m diameter and are hence preferred for bioprinting constructs with high cell density (e.g. cell aggregates) [7]. However, the efficacy of tissue fabrication by this printing modality is largely dependent on (i) physical and chemical properties of biomaterials used for cell printing to mimic tissue specific tissue ECM; (ii) shear-thinning properties to allow for fluidity within the printing nozzle for cell protection; (iii) cell-friendly cross-linking chemistry for post-printing stability and (iv) the ability to maintain the viability and functionality of embedded throughout the bioprinting process. This gave rise to the concept of creating structurally/ functionally sophisticated biomaterial formulations, often referred to as bioinks, which are aqueous formulations of polymers or hydrogel precursors that contain cells and biological factors.

There exists a volume of literature on various kinds of hydrogels used for extrusion based bioprinting [8]. Among them, gelatin methacrylamide (GelMA) is widely used for bioprinting owing to its biocompatibility and superior printability. GelMA is readily photocrosslinkable in presence of photoinitiators like Irgacure 2959 and Lithium phenyl-2,4,6-trimethylbenzoylphosphine [9]. We have reported previously that during photo-crosslinking of GelMA, excess reactive oxygen species (ROSs) are generated within HepG2 cells embedded in GelMA and inclusion of a free radical scavenger (FRS) molecule, such as AA (vitamin C) in the GelMA bioink could nullify the adverse effect of excess ROS produced inside the cells [10]. The use of antioxidants as photoprotectants, either alone or in combination, has been extensively documented in the literature. For example, it is reported that a combination of vitamins C and E is superior for UV protection to skin than an

identical concentration of vitamin C or E alone. This is due to the fact that when vitamin C and vitamin E interact synergistically, they provide enhanced antioxidant protection. Vitamin E scavenges active free radicals during photo-crosslinking and gets oxidized to form vitamin E radicals. In the presence of vitamin C, vitamin E radicals get reduced and thus both work synergistically [11, 12].

In this study, we utilized this synergic mechanism to formulate a novel photo-protective bioink for the fabrication of bioprinted liver construct using high density (20×10^6 cells ml^{-1}) of metabolically active primary hepatocytes. The bioink formulation consisted of GelMA as base polymer and a cocktail of anti-oxidants vitamin C (L-AA) and vitamin E (α -Toc) along with a photoinitiator. The bioink was evaluated for its rheological characteristics, cell compatibility and biocompatibility. The bioink was used to fabricate liver construct and the construct was subjected to histo-morphometric and functional analysis.

2. Materials

Gelatin (Type A, porcine, 175 Bloom), Irgacure 2959, Fluorescein diacetate (FDA), propidium iodide (PI), cell counting kit (CCK-8), minimum essential medium (MEM), and Iscove's modified Dulbecco's medium (IMDM) were obtained from Sigma, India. Fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin antibiotics were purchased from Gibco Invitrogen (India). Phosphate-buffered saline (PBS) was procured from TaKaRa (Shiga, Japan). The mouse subcutaneous fibroblast cell line (L-929) was procured from the American Type Culture Collection (USA) and human hepatocellular carcinoma (HepG2) cells were procured from the National Center for Cell Sciences (Pune, India).

2.1. Cell culture

Fibroblast (L-929) and hepatocyte (HepG2) cells were maintained in MEM supplemented with 10% FBS and 1% penicillin-streptomycin antibiotics in a carbon dioxide (CO_2) incubator set at 37 °C, 5% CO_2 , and 95% relative humidity.

Primary rat hepatocytes were isolated from male Wistar rats with prior approval from the Institutional Animal Ethics Committee (SCT/IAEC-406/JULY/2021/110) using a two-step perfusion method, as reported earlier [13]. Isolated hepatocytes were maintained in IMDM supplemented with 2% FBS and 1% penicillin-streptomycin antibiotics in a CO_2 incubator (New Brunswick, Germany) set at 37 °C, 5% CO_2 , and 95% relative humidity.

2.2. Synthesis of GelMA

GelMA was synthesized and characterized as previously reported [10, 14]. Gelatin was modified by reacting with methacrylic anhydride under optimum

temperature and pH. The reaction mixture was then dialyzed, lyophilized, and further sterilized by ethylene oxide gas at 37 °C.

2.3. Preparation of base GelMA

The base GelMA formulation was prepared by dissolving GelMA (5% w/v) in serum-free medium (MEM or IMDM) at 37 °C, which was later supplemented with 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) at a final concentration of 0.5% (w/v) as photoinitiator. GelMA hydrogels were prepared by exposing 250 µl of the GelMA bioink formulation to 365 nm UV light at intensity of 8–12 mW cm⁻². For the cell culture experiments, GelMA bioink formulations were prepared under aseptic conditions.

2.4. Phototoxicity analysis

To estimate the effect of UV damage on GelMA encapsulated cells by photo-crosslinking, intracellular ROS (iROS) were measured by DCFDA-cellular ROS assay (Abcam ab113851), as previously reported [12]. HepG2 cells at a concentration of 9×10^5 cells were mixed with 600 µl of GelMA solution to complete the bioink. The bioink solution (200 µl) was transferred to 24 well plate and cross-linked by irradiation with UV light at 365 nm for 3 min to encapsulate the cells within the GelMA hydrogel. Similarly, cell monolayer in 24 well plate exposed to UV for 3 min were considered as positive controls, and normal cells without UV treatment were considered as negative controls. After UV exposure, cells from the GelMA hydrogel were harvested using collagenase A treatment. The control cells were treated with collagenase. The retrieved cell suspension was washed with PBS, resuspended in PBS (500 µl), and transferred into a black-clear 96 well plate and 2', 7'-dichlorofluorescein diacetate formed due to iROS activity was quantified by measuring the fluorescence at excitation/emission wavelengths of 485 nm/535 nm using a spectrofluorometer (Synergy H1, Biotek, USA). The percentage of ROS activity was calculated for the cells retrieved from GelMA and the positive control by normalizing with the negative control.

2.5. Photoprotective analysis of antioxidant cocktail loaded GelMA

An antioxidant cocktail was then added to the base GelMA bioink formulation. Two antioxidant vitamins, AA and α -Toc were used. The concentration of AA selected was 3.4 mM as previously reported [10]. The concentrations of α -Toc were 25 µM and 100 µM. A stock concentration of 1 M α -Toc was prepared in pure ethanol, diluted in PBS, and added to the GelMA formulation to obtain final concentration of 25 µM and 100 µM α -Toc. The UV protective property of GelMA-antioxidant formulations was analyzed by measuring iROS production by cells

encapsulated within the hydrogels, as discussed in the previous section. The percentage of ROS activity in cells within each GelMA-antioxidant formulation (3.4 mM AA, 25 µM α -Toc and 100 µM α -Toc) was calculated by normalizing with that of the base GelMA formulation.

2.6. Preparation of GelMA-antioxidant (GelMA-AO) formulation and evaluation

The GelMA-AO formulation was prepared at final concentrations of 5% GelMA, 3% gelatin, 0.5% irgacure 2959, 3.4 mM AA and 100 µM α -Toc. The Gelatin was added to impart thermosetting properties, enabling the bioink to be printed within the temperature range of 18 °C–20 °C. GelMA-AO hydrogel was prepared by crosslinking the solution under 365 nm UV light for 3 min.

2.6.1. In vitro cytotoxicity evaluation of hydrogel

The cytotoxicity of the GelMA-AO hydrogel was evaluated by direct contact and an extraction method based on ISO 10993-5.

The GelMA-AO formulation was preincubated at 37 °C and transferred to a 60 mm diameter sterile petri dish. The solution was crosslinked by exposing to 365 nm UV light for 3 min. The test sample for the direct contact test was prepared by cutting 4 mm discs from GelMA-AO hydrogel sheets. Ultra-high-molecular-weight polyethylene (UHMWPE) and organo-tin stabilized polyvinyl chloride were used as negative and positive cytotoxicity controls, respectively. Triplicate samples were placed on an L-929 monolayer and incubated at 37 °C for 24 h. The cells around the samples were examined under a microscope and cytotoxicity was graded based on the ISO 10993-5 standard.

Cytotoxicity was analyzed by exposing cells to the GelMA-AO hydrogel extract. L-929 cells were seeded in 96 well plate at a density of 1×10^5 cells/well and allowed to attain sub-confluency. Hydrogel discs with a thickness >1 mm was prepared using a Teflon mold. The extract was prepared by incubating the hydrogel discs in culture medium containing serum at an extraction ratio of 1.25 cm² ml⁻¹ in a shaking incubator at 37 °C for 24 h. The 100% extract was diluted with culture medium to obtain 50, 25 and 12.5%. GelMA-AO extract (100 µl) at each dilution was transferred to L-929 cells ($n = 8$) and incubated for 24 h. Cells in normal culture medium were considered as negative controls and cells treated with 1.3 mg ml⁻¹ phenol in culture medium were considered as positive cytotoxic controls. Cell morphology was examined microscopically for the response to extract treatment, and cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Cytotoxicity was determined by calculating the percentage cell viability expressed by cells treated with GelMA-AO extract

and the positive control after normalizing the values with that of the cell control.

2.6.2. HepG2 cell adhesion, viability and proliferation

The adhesion and morphology of HepG2 cells on the GelMA-AO hydrogel were studied using actin staining. HepG2 cells at a density of 3×10^5 cells/well were seeded on a GelMA-AO-coated cover glass placed on 12 well plate. Following culture for 72 h, culture medium was removed, and cells adhered to GelMA-AO were rinsed thrice with PBS and fixed using 4% paraformaldehyde for 30 min. The cells were permeabilized by treatment with 0.1% Triton-X 100 for 3 min and incubated with rhodamine-phalloidin (1:100 dilution in PBS) for 15 min. Cell nuclei were counterstained with Hoechst 33 258 ($0.05 \mu\text{g ml}^{-1}$ in PBS). The cells were observed under a fluorescence microscope (Leica DMI 6000, Switzerland) using filters N21 (red emission) and A (blue emission).

HepG2 cells at a density of 3×10^5 cells were encapsulated in 250 μl of GelMA-AO hydrogel by UV exposing for 3 min and cell viability was analyzed by live/dead staining immediately after photocrosslinking (0 h) and after day 1 (24 h). Cells were incubated in serum free medium containing 0.01% FDA and 0.005% PI. The cells were observed under a fluorescence microscope using I3 (green emission for live cells) and N21 (red emission for dead cells) filters.

Also, the proliferation of HepG2 cells (3×10^5 cells) encapsulated in 250 μl of GelMA-AO hydrogel was analyzed using cell counting kit—8 (CCK-8) on days 1 and 7. The culture medium was replaced with the CCK-8 medium (1:10 dilution in serum-free medium) and incubated for 2 h. The CCK-8 medium was collected in a fresh 96 well plate and the absorbance were measured at 450 nm using a multi-well plate reader (Biotek, USA). The percentage of cell activity was calculated by normalizing the absorbance value at day 7 with the average absorbance value obtained on day 1.

2.6.3. Rheology

Rheological properties of the GelMA-AO bioink formulation were analyzed using a rheometer (Anton Paar, Germany). Temperature sweep and viscosity variation of the pre-crosslinked GelMA-AO formulation were performed. Temperature sweep tests were conducted at varying temperatures (24°C – 8°C) to determine the physical gelation temperature of GelMA-AO formulation, and the viscosity variation of GelMA-AO solution at 20°C was measured at variable shear rate (1 – 1000 s^{-1}) using a parallel plate PP25, 0.5 mm measuring distance to study the shear-thinning behavior of GelMA-AO formulation. To investigate the UV photo-polymerization of GelMA-AO formulation, the sample were exposed to 365 nm UV light source Omnicure 1000 (Lumen Dynamix LDGI) at 20°C under a constant shear strain of 1%,

and the variation in storage modulus was recorded up to 200 s.

2.6.4. 3D printing

The printability of GelMA-AO formulation was primarily assessed by filament extrusion test followed by printing different patterns like square, triangle and zigzag to study the width size of the extruded filament, the ability of the bioink to print and maintain different angles. Similarly, a circle pattern was also printed to study the ability of bioink formulation to print curvature. A square grid multilayer pattern with internal perpendicular filaments with outer dimensions of $10 \times 10 \times 2 \text{ mm}$ was also printed to evaluate the shape fidelity of the bioink formulation. All the patterns were created using 3D BioCAD (regenHU, Switzerland) software and G-codes were generated to print at a speed of 10 mm s^{-1} at 20°C . Printing experiments were performed using extrusion print head of 3D printer (3D Discovery, RegenHU, Switzerland) with nozzle of size $410 \mu\text{m}$. Before initiating printing, the 5 ml cartridge containing the GelMA-AO solution was kept at 2°C – 8°C for 2–3 min to allow for physical gelation and the cartridge was loaded into a 3D printer and were printed based on the selected design. The printed multilayer constructs were cross-linked with 365 nm UV light for 3–5 min.

The surface morphology of 3D printed GelMA-AO constructs were analyzed by SEM (Model: Hitachi, S2400). The 3D printed samples were lyophilized, sputter coated with gold and observed under SEM. Images were captured and pore size was measured by image analysis using Fiji Image J software.

To study the stability of the 3D printed GelMA-AO hydrogel under physiological conditions, the constructs were incubated in MEM complete medium at 37°C in a CO_2 incubator for seven days. The initial weight (W_i) of the construct ($n = 4$) was measured on day 0, and the final weight (W_f) was measured after incubation on days (1, 3, and 7). The percentage of weight loss of the construct was calculated using the following equation:

$$\text{Weight loss (\%)} = \frac{W_i - W_f}{W_i} \times 100.$$

2.6.5. Subcutaneous implantation of 3D printed construct

The biocompatibility of the GelMA-AO hydrogel was evaluated in a rabbit model with prior approval from the Institutional Animal Ethics Committee of Bioneed India Private Limited (BIO-IAEC-4303). GelMA-AO constructs with a size of $10 \times 10 \times 2 \text{ mm}$ were 3D printed and used for subcutaneous implantation as per ISO 10993-6 biological evaluation of medical devices”- tests for local effects after implantation. Clinically used collagen (ColoPlug, India) of a similar size to the test sample was used as a control. Adult male New Zealand White rabbits weighing 2.3–2.4 kg were subjected to surgical procedures

under general anesthesia by intramuscular injection using a mixture of xylazine (5 mg kg^{-1}) and ketamine (35 mg kg^{-1}).

The test item was implanted into the subcutaneous tissue on the right side by blunt dissection 25 mm apart from each implant, and the reference item pieces were implanted on the left side by blunt dissection 25 mm apart from each implant. A glass bead sterilizer was used to sterilize the surgical equipment. Subcutaneous pockets were made by blunt dissection, 25 mm from each site. The GelMA-AO hydrogel ($n = 4$) was implanted on the right side, and control collagen samples ($n = 4$) pre-soaked in sterile PBS were implanted on the left side. The skin incisions were closed using nonabsorbable suture materials. The animals were observed daily for mortality throughout the experimental period of 15 d. At the end of the observation period, animals were sacrificed by intravenous administration of sodium thiopentone. The implant sites, together with sufficient surrounding tissue, were collected and further processed for histopathological analysis. The tissue response at each implant site was determined, and the average score for the test and control groups was calculated. The average score for the control group was subtracted from the average score of the test group to obtain the histological evaluation score. The response to the test sample was graded as minimal or no-reaction (0.0–2.9), slight reaction (3.0–8.9), moderate reaction (9.0–15.0) or severe reaction (>15).

2.7. GelMA-AO bioink and optimization of high cell density 3D bioprinting

Liver construct was 3D bioprinted using GelMA-AO bioink (5% GelMA, 3% gelatin, 0.5% irgacure 2959, 3.4 mM AA and $100 \mu\text{M}$ α -Toc and primary rat hepatocytes isolated from Wistar rats). Bioink with two different cell concentrations, 1×10^6 cells/construct (1 M) and 5×10^6 cells/construct (5 M) was prepared. Bioprinted constructs were stabilized under UV irradiation and cultured in IMDM + 2% FBS medium in a CO_2 incubator at 37°C . Culture media were collected from the constructs and analyzed for albumin and urea production. Cell distribution inside the construct was assessed using hematoxylin and eosin (H&E) staining.

2.8. Functional evaluation of 3D bioprinted liver with high cell density

Culture media collected from bioprinted constructs (days 1, 3, and 5) were analyzed for urea and albumin secretion. The amount of urea released by the construct was estimated using a commercial urea assay kit (Biochain, Cat No: Z5030016) according to the manufacturer's instructions. The albumin secreted by the constructs was measured using a rat albumin ELISA Quantitation kit (Bethyl, Cat no: E111-125) according to the manufacturer's instructions.

The amount of albumin synthesized by the construct was determined from a standard curve obtained for rat albumin.

2.9. Statistical analysis

All experiments were performed in triplicate ($n = 3$). The results are presented as mean $\pm$ standard deviation, and significance was calculated using Student's *t*-test. Statistical significance was set at $P \leq 0.05$.

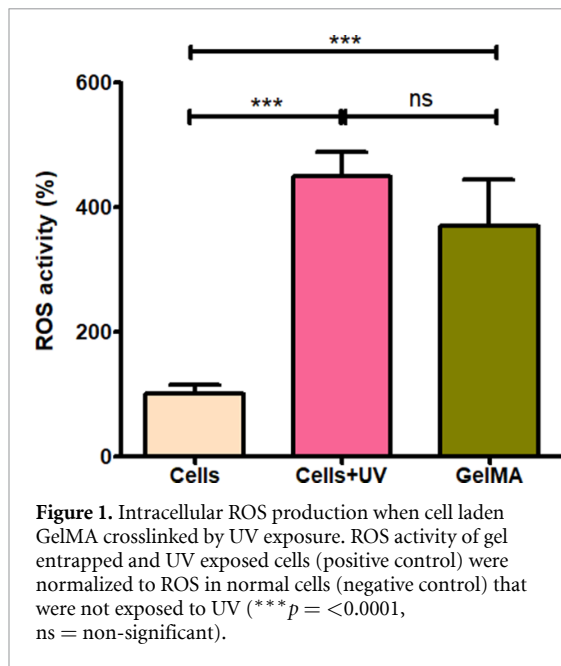
3. Results

3.1. Phototoxicity analysis in GelMA encapsulated cells

The formation of ROS in HepG2 cells exposed to UV light was measured to explain the phototoxicity. The iROS levels of HepG2 cells encapsulated in the base GelMA formulation were compared with those of normal cells (negative phototoxicity control) and cells exposed to UV (positive phototoxicity control). Figure 1 shows the ROS activity of the UV-exposed cells and cells encapsulated in the GelMA hydrogel. The UV exposed cells showed $450 \pm 16\%$ increase in iROS compared to normal cells without UV exposure. The GelMA entrapped cells also showed significantly higher ROS activity, reaching to $370 \pm 30\%$ compared to negative control.

3.2. Photoprotective effect of antioxidant loaded GelMA on cells

The photoprotective GelMA was formulated by optimizing the concentration of antioxidants and their subsequent effect on iROS production within encapsulated HepG2 cells. We have previously reported the standardized concentration of cytoplasmic protective agents by incorporating 3.4 mM AA into GelMA [10]. The effect of free radicals on the cell membrane may also affect cells. Hence, we evaluated two concentrations of α -Toc in GelMA (GelMA- α Toc $25 \mu\text{M}$ and GelMA- α Toc $100 \mu\text{M}$) and compared the intra cellular ROS activity of cells encapsulated in the GelMA hydrogel (figure 2). The result indicates that when compared with conventional base GelMA formulation, the cells in GelMA-AA only $38 \pm 1\%$ of ROS activity in base GelMA. Similarly, cells in GelMA- α Toc $100 \mu\text{M}$ also showed lower ROS activity of $57 \pm 3\%$ with respect to GelMA alone, whereas GelMA- α Toc $25 \mu\text{M}$ did not show a significant difference. Hence, the antioxidant cocktail was formulated with 3.4 mM AA and $100 \mu\text{M}$ α -Toc (GelMA-AA- α Toc $100 \mu\text{M}$) and analyzed for ROS activity. The results confirmed that the combination of AA and α -Toc had better efficiency in photoprotection, as evidenced by ROS activity of only $29 \pm 0.4\%$ compared to GelMA.



3.3. Bioink evaluation of GelMA-Antioxidant (GelMA-AO) formulation

3.3.1. In vitro cytotoxicity evaluation of hydrogel

In vitro cytotoxicity analysis of the GelMA-AO hydrogel based on ISO 10993-5 by direct contact with L-929 fibroblast cells did not show cytotoxic reactivity under or around the sample. Hence, it achieved a score of grade 0, which was considered non-cytotoxic (figure 3(a)). The negative control showed non-cytotoxic reactivity and the positive control showed severe cytotoxic reactivity.

The cytotoxicity due to the potential leachant from GelMA-AO hydrogel was analyzed by testing the extract method using L929 cells. After treatment with various dilutions of GelMA-AO extract, cells showed non-cytotoxicity with a cytotoxicity grade 0 based on ISO 10993-5. This was comparable to the morphology of cells cultured in the normal medium (cell control) (figure 3(b)). Cells treated with diluted phenol showed severe grade 4 cytotoxicity. To quantify the cytotoxicity, the MTT assay was performed soon after observation of cells at the end of the treatment time. The activity of cells treated with dilutions of GelMA-AO extract and phenol was normalized to that of the cell control. Cytotoxicity was calculated as a percentage of cell activity (figure 3(c)). The 100%, 50%, and 25% extracts of GelMA-AO showed mean cell activity of more than 100%, and there was no statistically significant difference compared to cell control. On the other hand, the cells treated with dilute phenol, which is considered a positive cytotoxic control, showed severe cytotoxicity with a Grade 4 score and <20% cell activity.

3.3.2. HepG2 cell adhesion, viability and proliferation

Staining of cytoskeletal structural proteins in HepG2 cells cultured on GelMA-AO hydrogel showed that

the hydrogel facilitated cell attachment. The cells showed a morphology on the gel surface and were evenly distributed (figures 4(a) and (a')). The viability of HepG2 cells encapsulated in the GelMA-AO hydrogel was analyzed by live-dead staining showed that cells were viable immediately after cross-linking, confirming the safe photocrosslinking process (figure 4(b)). The viability of HepG2 cells was analyzed after 24 h of encapsulation (figure 4(c)), confirming the suitability of GelMA-AO as a cyto-compatible hydrogel. The proliferation of HepG2 cells encapsulated in the GelMA-AO hydrogel analyzed by the CCK-8 assay is shown in figure 4(d). The cell activity measured on the 7th day after cell encapsulation was normalized to that measured on day 1. Assuming that the cells will multiply at least once in 7 d, the results substantiate the evidence for cell proliferation.

3.3.3. Rheology

The effects of temperature, shear rate, and time on the physical properties of the bioink were investigated by studying the rheological properties of GelMA-AO formulations. It was observed that the viscosity was inversely proportional to the temperature. A temperature sweep analysis of GelMA-AO formulation conducted in the range of 24 °C–8 °C is shown in figure 5(a). The gelation point is a sudden increase in viscosity with variation in temperature. GelMA-AO formulations showed a sudden increase in viscosity in the temperature range 18 °C–20 °C suggesting its gelation point. During extrusion, it is important that the bioink containing live cells be extruded through a small orifice of the nozzle without applying excessive shear to avoid the loss of cell viability. For this reason, it is necessary for the bioink to exhibit shear thinning behavior, which refers to the non-Newtonian behavior of fluids, so that the viscosity of the fluid decreases under shear. This shear-thinning behavior of the GelMA-AO formulation was evident from the viscosity curve shown in figure 5(b). The photo-crosslinking analysis under UV irradiation at a temperature above the gelation temperature of the GelMA-AO formulation is shown in figure 5(c), which shows the variation of storage modulus with respect to increasing UV exposure time from 50–200 s which clearly indicates that the GelMA-AO solution undergoes crosslinking.

3.3.4. 3D printing

Filament extrusion test shows the extrudability of GelMA-AO formulation under pressure which is an essential criterion for qualifying a bioink (figure 6(a)). Patterns printed using formulation suggest that the width of the extruded filament was same as that of the nozzle size (410 µm inner diameter) used for printing with no shrinkage or swelling of the filament (figure 6(b)). The 3D printed multilayer

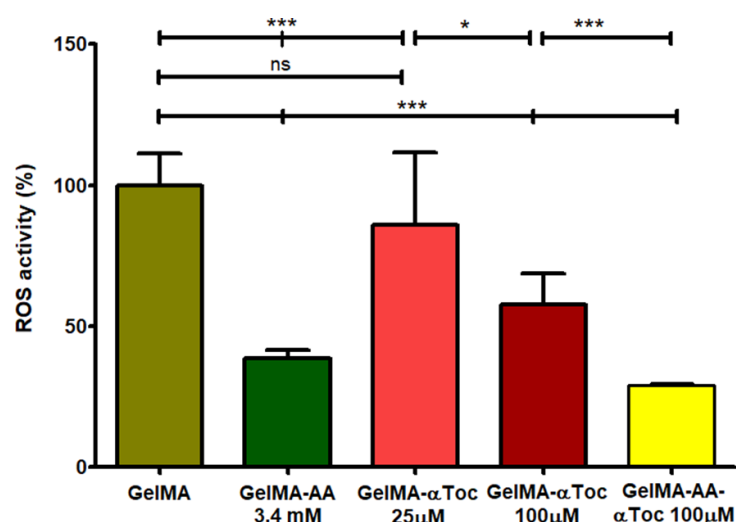


Figure 2. Photoprotective property of antioxidant loaded GelMA formulations. ROS activity in cells encapsulated in GelMA was considered as 100%. ROS activity in antioxidant loaded GelMA hydrogels were compared with GelMA. Combination of AA and α -Toc showed lesser ROS activity compared to all the formulations individually taken. ($n = 4$, *** $p < 0.001$, ns = non-significant).

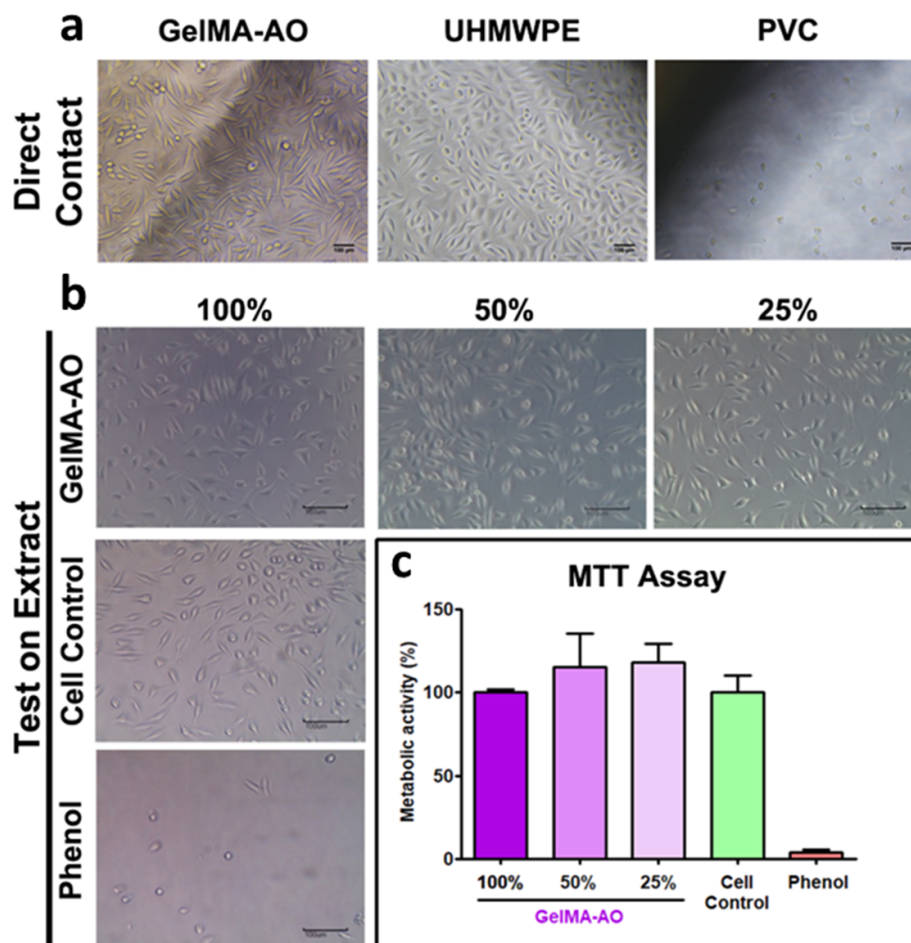


Figure 3. Cytotoxicity analysis of GelMA-AO hydrogel. (a) L929 cells after 24 h direct contact with GelMA-AO, negative control (UHMWPE) and positive control (1.3 mg ml^{-1} phenol). (b) Test on extract analysis showing L929 cells after 24 h treatment with dilutions of GelMA-AO extract, Cell control (culture medium) and dilute phenol. (c) Graph showing cell activity of cells determined by MTT assay at the end of test on extract.

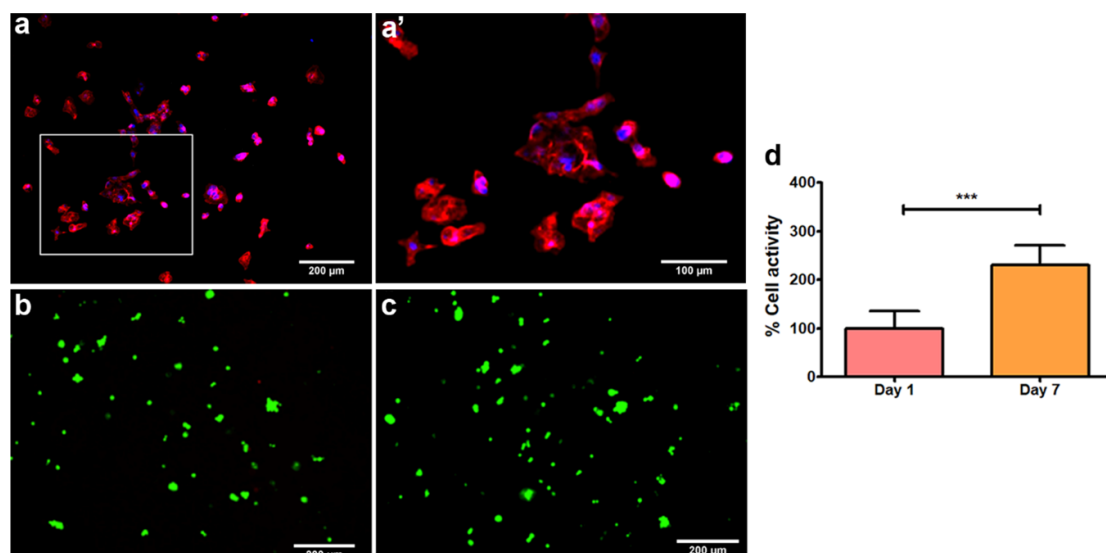


Figure 4. Cytoskeletal staining (red) of HepG2 cells adhered on GelMA-AO hydrogel. (a) Cells distributed over the surface showing adhesion. (a') Magnified area showing cell morphology and spreading. Cell nuclei were counter stained (blue) to identify the cells. Viability of HepG2 cells encapsulated in GelMA-AO hydrogel showing viability (green) at (b) 0 h and (c) 24 h of encapsulation. (d) Proliferation of HepG2 cells encapsulated in GelMA-AO bioink (***) $p = <0.0001$.

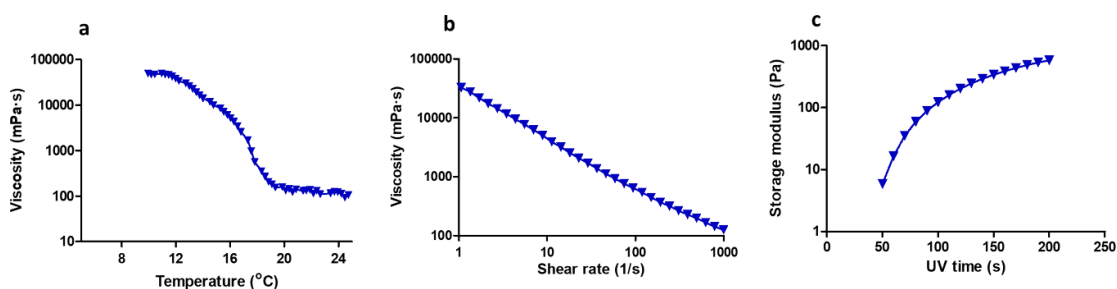


Figure 5. Rheological behavior of GelMA-AO bioink formulation (a) temperature sweep, (b) viscosity curve and (c) photocrosslinking.

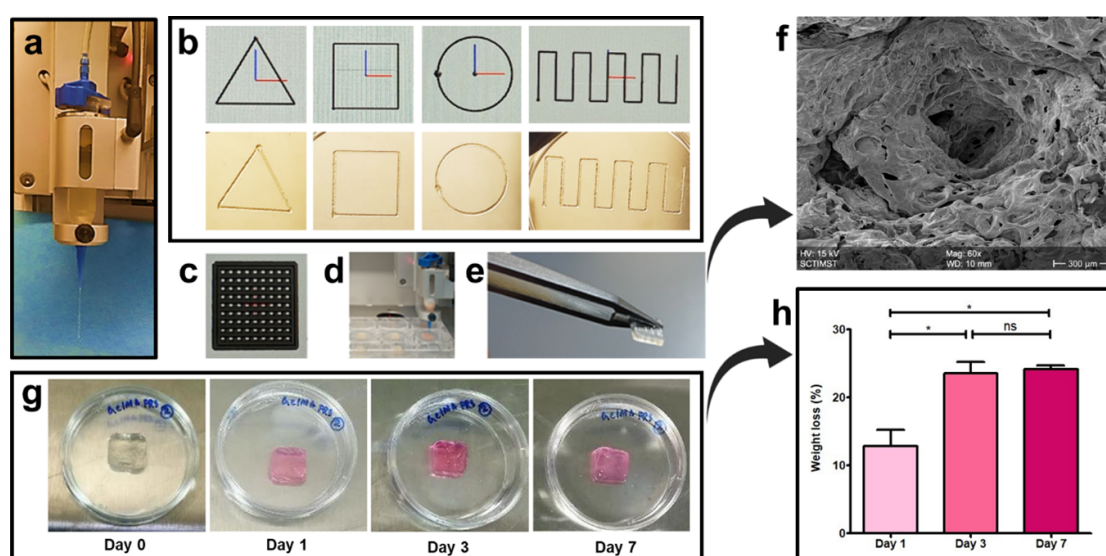


Figure 6. Three-dimensional printing of constructs with GelMA-AO formulation (a) The extrusion of GelMA as filament at appropriate pressure and temperature, (b) Printability of GelMA: first row represent various designs and second row shows corresponding printed GelMA, (c) 3D CAD design of multilayer construct, (d) 3D printing inside multiwell plate (e) 3D printed GelMA-AO construct, (f) SEM image of GelMA-AO construct showing porous nature, (g) 3D Printed GelMA-AO constructs incubated in culture medium for 0, 1, 3, and 7 d and (h) graph depicts the percentage weight loss of 3D printed constructs on day 1, 3 and 7 relative to day 0.

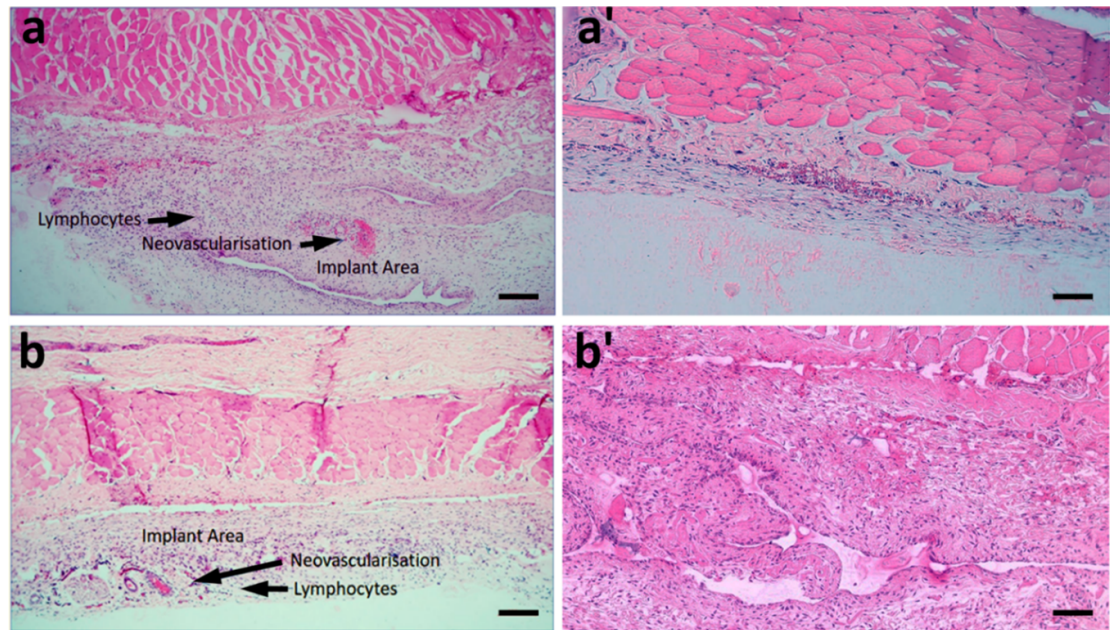


Figure 7. H&E staining of subcutaneous implanted 3D printed GelMA-AO construct. (a) and (a') showed the control sample under 5 $\times$ and 10 $\times$ magnification. Similarly, b and b' shows images of GelMA-AO at 5X and 10X magnification. Scale bar a&b = 200 μ m, a'&b' = 100 μ m.

square grid constructs also showed the same size as the CAD design (figures 6(c)–(e)).

3D printed GelMA-AO constructs observed under SEM showed filamentous and porous nature (figure 6(f)). The pore size measured by image analysis showed that the average pore size was 102 ± 20 μ m.

The stability of the 3D printed GelMA-AO construct under physiological conditions was analyzed based on weight loss over time. The constructs were stable in culture medium at 37 $^{\circ}$ C and maintained their shape over a period of 7 d (figure 6(g)). The quantitative analysis of weight loss depicted in figure 6(h) shows that there was a slight reduction of $9.6 \pm 6\%$ on day 1 and, reaching up to $20.2 \pm 6\%$ on day 3. On subsequent days, there were no further weight loss observed until day 7 ($20.7 \pm 6\%$).

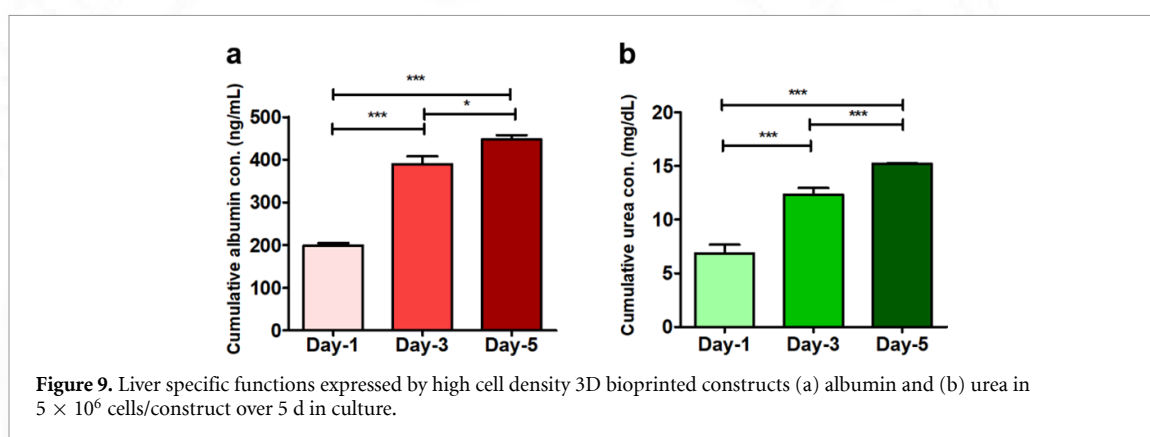
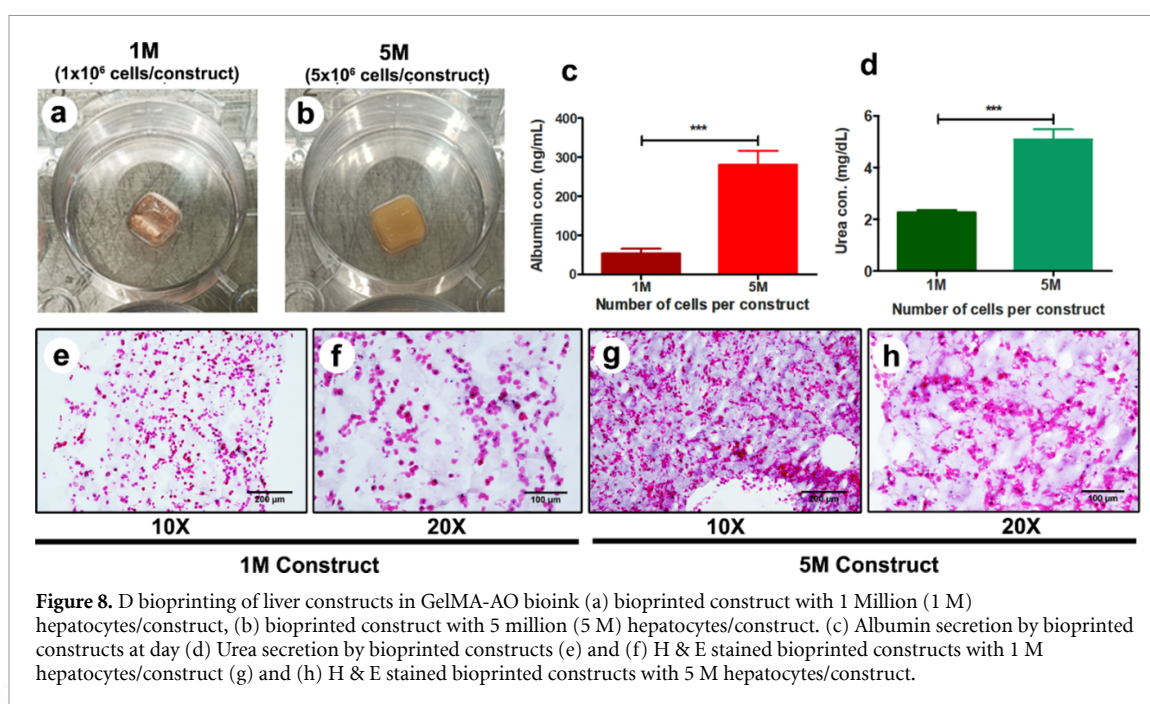
3.3.5. Subcutaneous implantation of 3D printed constructs

Subcutaneous implantation of 3D printed GelMA-AO construct did not show any signs of mortality or morbidity. All animals survived until the completion of the study. There were no treatment-related changes in body weight. There were no gross pathological observations during necropsy in any tissue, including at the implantation sites (subcutaneous tissue). Histological analysis of the tissue around the implant showed lymphocytic infiltration at implantation sites with a score of 1 in collagen control and scores of 1–2 in the GelMA-AO test item. Figures 7(a) and (a') shows macrophage infiltration around both the control and test sample implantation sites achieved a score of 1–2. The host tissue response in terms

of neovascularization in both control and GelMA-AO implantation sites is shown in figures 7(a) and (a'). Neovascularization was graded from 1 (minimal capillary proliferation, 1–3 buds) to 2 (group of 4–7 capillaries with supporting fibroblastic structures). The response at each implantation site was determined and the average score for the control and GelMA-AO sites was calculated. The average score for the control site was subtracted from the average score of the test site to obtain the histological evaluation score of the test item. The results showed that histological evaluation score for GelMA-AO was 0.6 (table 1: supplementary data). Thus, subcutaneous implantation of 3D Printed GelMA-AO hydrogel in a rabbit model resulted in minimal or no reaction to the subcutaneous tissue when evaluated at the end of 14 d.

3.4. Optimization of high cell density 3D bioprinting

The cell number for high cell density bioprinting was optimized at 4×10^6 cells ml^{-1} and 20×10^6 cells ml^{-1} concentrations of rat hepatocytes. A 3D construct of size $10 \times 10 \times 2$ mm requires approximately 250 μ l of bioink, which produces a construct with 1×10^6 cells (1 M) and 5×10^6 cells (5 M) as shown in figures 8(a) and (b). Albumin and urea synthesis are indicators of liver-specific functions in 3D bioprinted constructs. Figures 8(c) and (d) show that there was a significant increase in albumin and urea secretion by the 5×10^6 cells/construct compared to the 1×10^6 cells/construct. Albumin secretion was approximately 5-fold in 5 M constructs (281 ± 20 ng ml^{-1}) compared



to 1 M construct (53 ± 7.2 ng ml⁻¹), indicating maintenance of protein synthesis in high cell density constructs. The urea secretion in 5 M constructs (5 ± 0.2 mg dl⁻¹) was 2.5-fold compared to 1 M construct (2.2 ± 0.05 mg dl⁻¹). As the cell concentration increased, it was expected to have an enhanced function.

Histological analysis by H&E staining of 3D bioprinted cells laden with 1 M and 5 M liver constructs showed even cell distribution. The cells in the 1 M constructs were dispersed and spread out with minimal cell-cell contact (figures 8(e) and (f)). Interestingly, the 5 M construct showed more cell-cell interactions and orientations (figures 8(g) and (h)). Since the 5 M construct has a higher cell density, the hydrogel content was lower and more cell-cell interactions were evident. Based on the functional and histological evaluation, it can be deduced that 3D bioprinted constructs with 5×10^6 cells/construct outperformed those with 1×10^6 cells/construct, and

the 5 M construct was selected for bioprinting high cell density constructs for extended duration.

3.5. Functional evaluation of 3D bioprinted constructs with high cell density

The functional performance of the high cell density (5 M) 3D bioprinted constructs in exhibiting liver-specific characteristics was confirmed by analyzing albumin and urea secretion over an extended period of 5 d (figures 9(a) and (b)). Both albumin and urea secretion showed a linear increase over 5 d in culture.

4. Discussion

GelMA which forms covalently cross-linked hydrogels under UV light ($\lambda = 365$ nm) exposure in the presence of a photoinitiator, is a widely used hydrogel in bioink formulations for 3D bioprinting. GelMA bioink has been studied for the fabrication of cardiac, skeletal, neural, bone, cartilage, and

skin tissues because of its excellent flow properties, biocompatibility, and controllable biodegradability [10, 15]. After 3D bioprinting, the cell-laden constructs were exposed to ultraviolet (UV) light. However, exposure to UV light may damage cells either by direct absorption of cellular DNA or indirectly via photosensitization reactions. During the photo-crosslinking of GelMA, a significant number of free radicals are produced during the radical-initiated crosslinking process. It is well established that exposure to UV light has a physiological impact by forming ROSs inside cells [16, 17]. In this study, we cross-linked GelMA by exposure to UV light at a wavelength of 365 nm and evaluated its phototoxicity to cells. We previously showed that the percentage of ROS formed in cells after UV crosslinking, results in extensive intracellular ROS production. The study also showed that the addition of 3.4 mM AA, a well-known FRS or antioxidant, to the GelMA bioink formulation offered photoprotection to the bioprinted parenchymal constructs with HepG2 cells by nullifying the UV radiation produced damage [10]. Similar experiment was also done in this study as control and shown in figure 1. This result was in line with that of our previous study, in which cells encapsulated with GelMA produced significantly higher ROS levels than cells not exposed to UV light. When we consider the 3D bioprinting of tissues with high metabolic activity, viability and functional performance must be maintained. In this study, we imparted a photoprotective property by formulating a cocktail of antioxidants such as AA and α -Toc. The role of these antioxidants in the bioink formulation was to enhance photoprotection during the crosslinking of liver constructs with metabolically active primary rat hepatocytes at high cell density. α -Toc is one of the extensively studied lipophilic molecules with antioxidant properties, and it has been reported that 100 μ M of α -Toc provides a protective effect against UVAI (340–400 nm)-induced DNA damage in keratinocytes *in vitro* [18]. Hence, α -Toc is considered a protective agent in topical sunscreens to prevent damage from sunlight. Based on previous reports, two concentrations of α -Toc (25 μ M and 100 μ M) were selected for this study and were included in the base GelMA formulation. Figure 2 shows that GelMA- α Toc 100 μ M significantly reduce the accumulation of intracellular ROS. We have previously reported that GelMA with 3.4 mM AA shows radical scavenging properties in bioinks. Here, we modified the formulation to enhance the photoprotective ability of GelMA for bioprinting liver constructs with high cell density. Analysis of ROS by cells encapsulated in GelMA loaded with a cocktail of AA (3.4 mM) and α -Toc (100 μ M) showed that the combination of antioxidants GelMA-AA- α Toc 100 μ M had a significant influence in reducing intracellular ROS compared to either antioxidant used alone. The synergistic interaction between AA and α -Toc was evident

in the outcome that resulted in better photoprotection when used in combination [12, 19].

In order to 3D bioprint liver construct with a maximal cell load and a minimum amount of matrix, 5% GelMA was used. It is also known that GelMA at a concentration of ≤ 5 w/v % is suitable for cell-laden constructs [20]. Low concentrations of GelMA affect 3D printability because of its poor viscosity and slow gelation rate. Addition of gelatin to 5% GelMA can improve the printability properties when it is used as a bioink. Therefore, we arrived at an optimized GelMA-AO formulation containing 5% GelMA, 3% gelatin, 3.4 mM AA, 100 μ M α -Toc and 0.5% irgacure.

To qualify the formulation for use as a bioink, it is essential to know its cytocompatibility [21, 22]. Cytocompatibility analysis of the GelMA-AO formulation was confirmed by cytotoxicity, cell adhesion, cell viability after encapsulation, and proliferation, as shown in figures 3 and 4. The actin filaments within the cells showed a direct evidence that the hydrogel promotes cell attachment. Bioink components influence its ability to be extruded in a consistent manner as well as maintenance of its structural integrity [23]. The rheological analysis of GelMA-AO revealed its thermoresponsive properties and enabled 3D printing at 18 °C–20 °C (figure 5(a)). Extrusion under pneumatic pressure might cause severe shear during bioprinting, which would affect cell viability [21]. Hence, one of the most important characteristics of bioinks is their shear-thinning behavior. The GelMA-AO formulation exhibited excellent shear-thinning behavior, as shown in figure 5(b). The addition of antioxidants may hamper crosslinking ability. Dynamic crosslinking analysis revealed an increase in the storage modulus with respect to the duration of UV exposure. Crosslinking within a duration of <200 s indicated that the antioxidants did not extensively affect the crosslinking property of the formulation (figure 5(c)).

Printability of a bioink is an important parameter to be considered while standardizing the bioink as the bioprinted product must mimic both cellular architecture and shape. The ability to extrude is a basic requirement for printability. A bioink formulation should be flowable and must be able to deposited precisely in terms of spatial, temporal, and volumetric control. Porosity of scaffold is important in tissue engineering as the pore size influences both nutrient and oxygen transfer for the survival, proliferation of cells and also in vascularization [24, 25]. It has been previously reported that pore size of 10% GelMA ranged 102 $\pm$ 20 μ m in diameter indicating suitable microenvironment for cell viability proliferation and function. The morphology of 3D printed GelMA-AO construct showed both macro and micro pores (figure 6(f)). Stability of the printed construct during *in vitro* maintenance under physiological conditions is another crucial factor. During the initial days, the observed reduction in weight could be due to

the release of gelatin from the construct [10]. Release of gelatin does not affect the shape or size of the constructs. Thus, it can be concluded that the 3D printed construct with GelMA-AO formulation was stable under physiological conditions (figures 6(g) and (h)).

The biocompatibility evaluation studies the local effects on the surrounding tissue (number and distribution of inflammatory cells, vascularity of fibrous capsule, granuloma, fatty infiltration, material debris, endothelialization, and presence of necrosis) following the implantation of biomaterials. We analyzed the biocompatibility of the GelMA-AO hydrogel in a rabbit model according to ISO 10993-6. When implanting hydrogels *in vivo*, a higher number of macrophages around the implant site can influence material degradation. Lymphocyte reactions to polymeric implants can also have negative effects and reduce biocompatibility [26]. In our experiment, collagen was used as the control material. When examined histologically (figure 7), the cell response in terms of macrophage infiltration at both control and test implantation sites was graded to a score of 1–2. Lymphocytic infiltration at the control implantation site achieved a score of 1 and the test implantation site achieved a score of 1–2. Vascular network formation at the implant site is critical for determining biocompatibility [27]. Neovascularization refers to the formation and growth of new blood vessels through several vascular processes, namely, vessel formation, sprouting, maturation, stabilization, remodeling, and specialization [28]. The tissue response in terms of neovascularization was graded as 1 (minimal capillary proliferation, focal, 1–3 buds) to 2 (group of 4–7 capillaries with supporting fibroblastic structures) at both the control and test item implantation sites. Thus, subcutaneous implantation of the 3D Printed GelMA-AO hydrogel can be considered to have minimal or no reaction to the subcutaneous tissue when evaluated at the end of 14 d.

The main requirement for 3D bioprinting of liver constructs is the fabrication of liver tissue that mimics the native tissue and exhibits liver-specific functions. Tissue engineering of liver tissues remains a challenge in developing functional constructs for clinical application and *in vitro* tissue models [29]. Some of the limitations of conventional tissue engineering can be overcome using 3D bioprinting. The advantage of 3D bioprinting is its ability to spatially distribute cells within the construct and to design the vasculature within the construct [30]. To achieve the highest level of functional performance in bioprinted constructs, it is necessary to mimic the cell arrangement and microenvironment of the native tissues. The liver tissue is made up of 70% parenchymal cells, 30% non-parenchymal cells, and fewer ECM components [31]. Hence, it would be preferable to consider the high cell density of hepatocytes and a low ECM compartment in biofabricated tissue. In this study, we designed a grid pattern of 1×1 cm

with an interfilament distance of 1 mm for efficient mass transfer. The bioink contained low (5% w/v) concentration of GelMA to accommodate high cell density. Dvir-Ginzberg *et al* reported high cell density bioprinting of liver constructs using alginate as a bioink [32]. Their study proved that a higher cell density of 5 million cells cm^{-3} maintained higher cell viability than those with low cell density. Here, we used GelMA-AO bioink to fabricate 5 million cells per construct with dimensions of $10 \times 10 \times 2$ mm, which is equivalent to 50 million cells cm^{-3} when extrapolated from a previous report. Histological analysis of cells in the 5 M cells/construct showed more cell-cell contact than the low cell density constructs. Albumin and urea synthesis analyzed 24 h after bioprinting showed 5-fold and 2.5-fold production, respectively, compared to the 1 M construct (figure 8). The 5 M constructs were further evaluated for an extended duration of 5 d, showing a linear increase in albumin and urea synthesis, substantiating the reason for improved functional performance in the high cell density construct, as shown in figure 9. Collectively, the results of this study provide evidence for the formulation of a photoprotective bioink for high cell density bioprinting of liver constructs.

5. Conclusions

This study reports the use of an antioxidant cocktail-loaded GelMA-based bioink with photoprotective properties for 3D bioprinting of liver constructs with high cell density. The results showed that the antioxidant cocktail system improved the radical scavenging properties of both antioxidants alone. Additionally, the 3D printed construct printed using the novel GelMA-AO formulation evaluated by subcutaneous implantation in a rabbit model confirmed its biocompatibility. Three-dimensional bioprinted constructs with a high density of hepatocyte cells showed improved functionality and cell organization compared to bioprinted constructs with low cell density. Our research proposes a potential method to overcome the phototoxicity of crosslinking bioprinted constructs using GelMA-AO bioink. The method and results presented herein will be useful for fabricating transplantable liver constructs that resemble native tissues.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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Author contributions

Conceptualization, P R Anil Kumar; Formal analysis, Anupama Sekar J; Funding acquisition, Shiny Velayudhan and P R Anil Kumar; Methodology, Anupama Sekar J and P R Anil Kumar; Project administration, P R Anil Kumar; Writing—original draft, Anupama Sekar J; Writing—review & editing, Shiny Velayudhan and P R Anil Kumar.

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